SUBSTRATE-MODIFIED

SULPHATASE A

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ERRATA

Page 3, line 10 from bottom. 'chondriotin' should read 'chondroitin'.

Page 4, Table 1. The heading should read as follows:

SULPHATE-CONTAINING COMPOUNDS

Types of sulphate esters and related compounds which occur naturally, together with typical examples thereof.

Page 4, Table 1, line 10. 'Carbohydate' should read 'Carbohydrate'.

Page 27, line 7. 'effected' should read 'affected'.

Page 33, line 15. 'hydrolysed' should read 'hydrolyzed'.

Page 37, line 2. 'monitered' should read 'monitored'.

Page 42, last line. Insert a hyphen in 'chloroform methanol'.

Page 71, Table 7. The heading should read as follows:

ELECTROPHORESIS OF OX LIVER SULPHATASE A

The electrophoresis of the native and neuraminidase-treated enzymes was carried out in SDS-polyacrylamide gels as detailed in the text and summarised in the Table. At least four gels were run under each set of conditions and the numbers in the brackets give the number of gels scanned to estimate the amounts of protein present in each of the bands corresponding to the two subunits of the enzyme.

Page 87, Table 8. The heading should read as follows:

EXTENT OF THE SUBSTRATE-INDUCED INACTIVATION OF SULPHATASE A UNDER VARIOUS CONDITIONS

The reaction mixtures contained 50 mM nitrocatechol sulphate at the pH specified in the Table and were incubated for 45 min at 37°C. The amount of native enzyme remaining at 30 min was estimated from the slopes of the progress curves at that time compared with the initial slopes.

Page 90, Table 10. The heading should read as follows:

REPEATED MODIFICATION OF SULPHATASE A

Results of two independent preparations of substrate-modified sulphatase A in which the modification procedure was carried out twice.

Page 98, Table 13. The column heading '$t_{\frac{1}{2}}$' should read '$t_{\frac{1}{2}}$ (h)'.

Page 115, line 1. The sentence beginning 'Waheed and ...' should read 'Waheed and Van Etten (1980a) have similarly shown that the sulphatase A of rabbit liver bound to CNBr-Sepharose forms, on incubation with nitrocatechol sulphate, a substrate-modified enzyme which can subsequently be reactivated by SO_4^{2-}.'.

Page 127, last line. 'effected' should read 'affected'.

Page 130, Table 17. The heading should read as follows:

STEADY-STATE VELOCITIES OF REACTIVATED SUBSTRATE-MODIFIED SULPHATASE A

The velocities were measured as described in the text with either nitrocatechol sulphate or p-nitrophenyl sulphate as substrate. The results of two sets of experiments are shown. In the first, the velocities in 6 mM and 1.5 mM SO_4^{2-} are compared: in the second, the velocities at ionic strengths 0.1 and 0.2 are compared.

Page 160, Table 22. The heading should read as follows:

SPECIFIC ACTIVITIES OF THE PREPARATIONS OF SULPHATASE A USED IN THE SPECTROSCOPIC MEASUREMENTS

The activity was measured with nitrocatechol sulphate, under the standard conditions described in the text, before and after the spectroscopic investigations.

Page 168, Table 23. The heading should read as follows:

MOLECULAR WEIGHTS OF SULPHATASE A DETERMINED BY SEDIMENTATION EQUILIBRIUM UNDER VARIOUS CONDITIONS

Page 189, Table 25. The heading should read as follows:

^{35}S-CONTENT OF PREPARATIONS OF SUBSTRATE-MODIFIED SULPHATASE A

The first part of the Table shows the number of atoms of ^{35}S per monomer unit of substrate-modified

sulphatase A isolated from reaction mixtures containing nitrocatechol [^{35}S] sulphate. The values are corrected for a nonspecific adsorption of $^{35}SO_4^{2-}$. The second part of the Table shows the effects of various treatments on the amount of ^{35}S present in the modified enzyme.

Page 196, Table 26. The heading should read as follows:

ISOTOPE-EXCHANGE BETWEEN ^{35}S-LABELLED SUBSTRATE-MODIFIED SULPHATASE A AND SO_4^{2-}

Values are given, for two preparations of the enzyme, of their associated radioactivities before and after dialysis against K_2SO_4.

Page 197, Table 27. The heading should read as follows:

ISOTOPE-EXCHANGE BETWEEN SUBSTRATE-MODIFIED SULPHATASE A AND $^{35}SO_4^{2-}$

Values are given, for two preparations of the enzyme, of their associated radioactivities before and after dialysis against $K_2{}^{35}SO_4$.

Page 199, Table 28. The heading should read as follows:

ISOTOPE-EXCHANGE BETWEEN THE SULPHATE GROUPS IN VARIOUS SULPHATE ESTERS AND $K_2{}^{35}SO_4$

The Table shows the radioactivity associated with the sulphate ester after this had stood for 4 days in 10 mM $K_2{}^{35}SO_4$ at 5°C.

The work described in this thesis was undertaken in the Department of Physical Biochemistry of the John Curtin School of Medical Research, Australian National University between January 1978 and January 1981. The results given were obtained by the author alone with the exceptions noted below.

The circular dichroism spectra were measured by Dr. D.I. Marlborough.

Advice and aid in doing the peptide mapping was obtained from Dr. D.C. Shaw.

Sedimentation equilibrium studies were carried out with the assistance of Dr. A.B. Roy.

E. Prosser

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ABSTRACT

The substrate-induced inactivation of sulphatase A (aryl sulphate sulphohydrolase EC 3.1.6.1) when acting as an arylsulphatase is a characteristic feature of this enzyme which distinguishes it from the other known sulphatases. During the hydrolysis of aryl sulphates, such as nitrocatechol sulphate, a substrate-modified form of the enzyme is produced which has no activity when incubated with substrate alone but is activated in the presence of sulphate or certain other anions. Although the substrate-modified enzyme exists in an equilibrium with the native enzyme, the activation cannot be explained in terms of reversion of the substrate-modified to the native enzyme. Evidence indicates that the activity induced in the substrate-modified enzyme by these anions does not alter the modification.

The substrate-modified form of the ox liver sulphatase A has been prepared by incubation of the native enzyme at 37°C in 0.05 M nitrocatechol sulphate or nitrocatechol [^{35}S]sulphate, pH 7.5, and isolated by chromatography. This gives a preparation which contains approximately 90% of the substrate-modified enzyme. It is stable at temperatures near 0°C at concentrations greater than 0.1 mg ml^{-1}. In more dilute solution the ability of the enzyme to activate is slowly lost and at higher temperatures reversion to the native form is observed. The latter has a $t_{\frac{1}{2}}$ of about 5 hours at 37°C and pH 7.4.

This preparation of the substrate-modified enzyme was then characterized kinetically and physically in order to define the differences between it and the native enzyme. The response of the catalytic activity of the substrate-modified enzyme to changes in substrate and activator concentrations is consistent with the hypothesis that it has two binding sites, each able to bind either substrate or activator. When one molecule of each is bound an active complex is formed. The relative concentrations of substrate and sulphate determine the extent of activation but not the rate at which the maximum velocity is attained.

The formation of the active complex of the substrate-modified enzyme, as well as the substrate-induced inactivation, may involve a conformational change in the enzyme although direct evidence for this has not been obtained. The active form of the substrate-modified enzyme could not be isolated from the reaction mixture and, therefore, the detection of any conformational motion related to the activation is greatly hindered. Circular dichroism, fluorescence and ultra-violet spectra of the native and substrate-modified enzymes at either pH 7.4, where the enzyme exists as a monomer, or at pH 5.0, where it exists as a tetramer, gave no indication of any difference between the two forms of enzyme. Inactivation, therefore, does not appear to be associated with any major conformational change.

By using nitrocatechol [^{35}S]sulphate as substrate it was shown that the sulphate moiety is bound to the enzyme

when it is inactivated. The amount of ^{35}S present in the preparations of substrate-modified enzyme was dependent on the extent of inactivation, but the ratio of 1 mole ^{35}S per mole substrate-modified enzyme was constant. Although it is not known whether the binding of the sulphate is the cause of inactivation or is a consequence of it, the ^{35}S remained associated with the substrate-modified enzyme during activation and was lost when it reverted to the native form.

ABBREVIATIONS

ATP	adenosine triphosphate
BSA	bovine serum albumin
CD	circular dichroism
I	ionic strength
M	molecular weight
NAD	nicotine adenine dinucleotide
NCS	nitrocatechol sulphate
P_i	inorganic phosphate
R	gas constant
SDS	sodium dodecyl sulphate
T	temperature
UV	ultra-violet

v	velocity
v_o	initial velocity
cpm	counts per minute
dpm	disintegrations per minute

ω	angular velocity
$\bar{v}$	partial specific volume
η	viscosity
ρ	solution density
λ_{ex}	wavelength of excitation light
λ_{em}	wavelength of emitted light
λ_{max}	wavelength where maximum occurs

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1. INTRODUCTION

Although aryl sulphates were shown to be natural products as early as 1876, and the occurrence of enzymes (sulphohydrolases) which would hydrolyze sulphate esters were observed in the early 1900's, it was not until the 1950's that detailed studies of these enzymes were undertaken. Prior to this time work on the sulphohydrolases had revolved around determining their distribution and specificity. This led to a wide variety of sulphohydrolases, or sulphatases, being discovered and classified according to the compound(s) they hydrolysed but little attention was paid to purifying them or studying their kinetics. Even today the physiological importance of most of these enzymes is unclear and much emphasis is placed on trying to unravel their specificities and metabolic roles. This task has been aided by the purification of many sulphatases which has allowed accurate kinetic studies to be undertaken and has proven instrumental to the understanding of these enzymes.

When a partially purified preparation of sulphatase A (aryl sulphate sulphohydrolase EC 3.1.6.1) was isolated from ox liver in 1953 (Roy, 1953) the anomalous kinetics, which have since come to be recognised as a characteristic feature of sulphatase A acting as an arylsulphatase, were first detected. As will become evident in the following general introduction, the same type of kinetics is not found with other sulphatases nor is it found with sulphatase A when cerebroside sulphate is used as substrate. An explanation of why this arylsulphatase inactivates and

of the mechanism responsible for the inactivation and subsequent activation is therefore of interest in understanding the behaviour of this enzyme and from a more theoretical point of view.

1.1 REACTION CATALYZED BY SULPHATASES

The reaction catalyzed by the sulphohydrolases, commonly called sulphatases, is given below for the general case.

$$R\cdot OSO_3^- + H_2O \rightarrow R\cdot OH + SO_4^{2-} + H^+ \qquad (1.1)$$

A sulphate ester is hydrolysed to produce inorganic sulphate with the concomitant production of H^+. Sulphate esters are the product of the esterification of a hydroxyl-containing compound with sulphuric acid. They are generally isolated as salts; $R\cdot OSO_3^-M^+$, but under experimental conditions will be fully ionised and are therefore written as $R\cdot OSO_3^-$ in the above equation (1.1). The R group can be aliphatic, aromatic or heterocyclic and ROH can be either an alcohol, phenol or carbohydrate.

Sulphate esters were first shown to be natural products by Baumann in 1876 when he isolated phenyl sulphate from urine. He went on to demonstrate that the injection of a number of phenols into mammals is followed by the excretion of the corresponding aryl sulphates. It was subsequently proposed that the formation of these esters was part of the detoxification process for phenols as the sulphated form is more soluble in aqueous solvents and therefore more readily excreted. It has also been suggested, however, that the aryl sulphate is merely the end product

of the metabolism of these compounds and has not been produced specifically to aid in its excretion.

Sulphate esters are produced *in vivo* by the transfer of the sulphuryl group of 3'-phosphoadenylyl sulphate (3'-phosphoadenosine 5'-phosphosulphate, (PAPS)) to an acceptor compound, either an alcohol or phenol, to form a sulphate ester and adenosine 3'5'-bisphosphate (PAP). A sulphotransferase, one of a large group of apparently specific enzymes, is responsible for this transfer.

A wide variety of sulphate esters is known and is found in plants, microorganisms, molluscs and mammals. A list of the different types of sulphate esters and some naturally occurring examples are shown in Table 1.

Some examples of these compounds which are found in mammals and are of particular interest are given below. Tyrosine O-sulphate, an aryl sulphate, occurs in several peptides and is excreted as the amino acid in urine. Some polysaccharide sulphates are keratan sulphate, found in the cornea and skeletal tissue, heparan sulphate, found in liver and lung, and chondriotin sulphate, found in cartilage. Sulpholipids containing galactose 3-sulphate residues such as cerasine sulphate and phrenosine sulphate occur in the brain. Steroid sulphates such as androstenolone sulphate and oestrone sulphate are also found.

Apart from what may be a purely structural role of glycosaminoglycans the function of these sulphate esters is not clear. For some, roles have been inferred by their distribution or effects observed when the sulphate is

TABLE 1

SULPHATE ESTERS

Type of compound	Example
Alkyl sulphates	Propan-2-yl sulphate
Aryl sulphates	Indoxyl sulphate
Steroid sulphates	
Phenols	Oestrone sulphate
Primary alcohols	Cortisol 21-sulphate
Secondary alcohols	Cholesteryl sulphate
Carbohydate sulphates	
Primary alcohols	Galactosamine 6-sulphate
Secondary alcohols	Galactose 3-sulphate
Hydroxamic acid sulphates	Sinigrin
Thiosulphates	S-Sulphocysteine
Sulphamates	2-Sulphoamino-2-deoxyglucose
Sulphatophosphates	Adenosine 5'-sulphatophosphate

removed. For example, sulphate conjugates of androgens are produced in the adrenal and liver and, as the conjugates are biologically inactive, it has been postulated that they may serve as a means of storage. These sulphate esters as well as those of other steroids may also be metabolic intermediates. It has recently been suggested that sulphate esters are involved in the opiate receptor found in synaptic membrane as opiate binding was inhibited by cerebroside sulphatase (Law *et al*, 1979). Karlsson *et al* (1968, 1969) have suggested, on the basis of their distribution, that sulphatides may be involved in the Na^+ transport system. In studying the sphingolipids of bovine kidney these authors observed a high concentration of sulphatides in the outer part of the medulla where the corticosteroid-dependent Na^+ transport system is located. A correlation has also been found between the sulphatide content of the herring gull salt gland and Na^+, K^+-activated ATPase activity (Stoffel, 1971).

Spencer (1958, 1959) demonstrated that with several arylsulphatases (equation (1.1) where R is aromatic) it was the O-S bond which was cleaved in the hydrolysis. This was done by carrying out the reaction in $H_2{}^{18}O$ and isolating the liberated sulphate. As the ^{18}O was found in the inorganic sulphate the O-S bond must have been broken. Had the C-O bond been involved the ^{18}O would have been associated with the resulting phenol. Human liver sulphatase A, rat liver sulphatase C and the arylsulphatases of *Alcaligenes metalcaligenes* and *Asperigillus nidulans* were used in these studies. Logan and Warren (1969)

similarly showed that the O-S bond was cleaved in the hydrolysis of dehydroepiandrosterone sulphate by human placental steroid 3β-sulphatase. In contrast, Bartholomew *et al* (1977) and Cloves *et al* (1978) demonstrated that the primary and secondary alkyl sulphatases (equation (1.1) where R is aliphatic) from *Pseudomonas* cleaved the C-O bond. It is perhaps noteworthy that the chemical hydrolysis of alkyl sulphates proceeds through C-O bond cleavage in alkali (Batts, 1966) while aryl sulphates are hydrolysed predominantly through O-S bond cleavage in alkali (Spencer, 1958). Under acid conditions both exhibit O-S bond breakage.

Other related enzymes; sulphamatases, which hydrolyze the sulphamates of glucosamine and galactosamine (1.2), and phosphosulphatases, which hydrolyze the sulphatophosphate linkage of adenylyl sulphate and 3'-phosphoadenylyl sulphate (1.3), have also been found.

$$R\cdot NH\cdot SO_3^- + H_2O \rightarrow R\cdot NH_3^+ + SO_4^{2-} \qquad (1.2)$$

$$\begin{array}{c} O \\ | \\ -O-P-O\cdot SO_3^- \\ | \\ OH \end{array} + H_2O \rightarrow -O\cdot PO_3H_2 + SO_4^{2-} + H^+ \qquad (1.3)$$

1.2 SULPHATASES

The name sulphatase was first used by Neuberg and Kurono (1923) in connection with an enzyme they observed in preparations of the fungus *Asperigillus oryzae* which would remove the sulphate from potassium phenyl sulphate. Many such enzymes have since been found. A list of the sulphatases described in a review published in 1971 (Roy, 1971b) is given in Table 2. The validity of naming the enzymes in this manner and of dividing them into these categories has become questionable as possible physiological substrates are found and the specificities of the different enzymes studied. In several cases when such enzymes have been purified they have been found capable of hydrolysing a wide variety of sulphate esters, not only their originally observed substrate. Sulphatase A is a good example of this. Originally studied as an arylsulphatase, it has been more recently shown to be identical to cerebroside sulphatase and also to hydrolyse sulphate esters of simple carbohydrates such as glucose 3-sulphate, galactose 6-sulphate and ascorbate 2-sulphate. More surprisingly, it has also been shown to hydrolyse adenosine 3',5'-monophosphate (Uchida *et al*, 1981). Sulphatase B, another enzyme designated an arylsulphatase, will also hydrolyse oligosaccharides of chondroitin sulphate and keratan sulphate. It is thought that these compounds are the physiological substrates for sulphatase B but that the hydrolysis must be preceded by depolymerization of the glycoaminoglycan chain. Evidence obtained by Dolly *et al* (1972) suggests that in the rat

TABLE 2

Enzyme	Common Substrate
Alkylsulphatases	dodecyl sulphate pentan-3-yl sulphate
Arylsulphatases	
Type I sulphatase C	p-acetylphenyl sulphate
Type II sulphatase A	nitrocatechol sulphate
sulphatase B	p-nitrophenyl sulphate
Glycosulphatases	
Glucosulphatase	D-glucose 6-O-sulphate
Chondrosulphatase	chondroitin 4-sulphate
Cellulose polysulphatase	charonin sulphate
Cerebroside sulphatase	cerebroside 3-sulphate
Steroid sulphatases	
Oestrone sulphatase	oestrone sulphate
Androstenolone sulphatase	androstenolone sulphate
Aetiocholanolone sulphatase	aetiocholanolone sulphate
Cortisone sulphatase	cortisone 21-sulphate
Myrosulphatase	mustard oil glycosides e.g. sinigrin
Choline sulphatase	choline sulphate

liver sulphatase C is identical to oestrone sulphatase but some controversy exists as to whether this is actually the case and also as to the separate identity of steroid sulphatase. Other investigators, using enzymes from different sources, have interpreted their data as evidence that these are all individual enzymes. For example, French and Warren (1967) measured the steroid sulphatase and oestrone sulphatase activity in human placenta, and Zuckermann and Hagerman (1969) measured the oestrone sulphatase and arylsulphatase activity in rat kidney and came to this conclusion. The interpretation of data obtained with these enzymes is difficult as they are insoluble and have not yet been purified. Only indirect evidence has therefore been presented to support either of these hypotheses. Once homogeneous preparations of these enzymes have been isolated this question will be more easily answered.

Both the uniqueness of these enzymes in all sources and the restricted range of substrate(s) which is implied by Table 2 are therefore in doubt.

1.21 Arylsulphatases

The enzymatic hydrolysis of an aryl sulphate was first observed by Derrien in 1911. He found an enzyme present in an extract of the mollusc *Murex trunculus* which was capable of hydrolyzing indoxyl sulphate. Similar enzymes were subsequently observed in extracts from other sources and a large number of aryl sulphates were shown to act as substrates in this reaction. This can be seen from the list of sulphate esters hydrolysed by sulphatases given by

Fromageot in his review in 1936. Early work was concerned mainly with the distribution of the enzymes. Phenyl sulphate and 1-naphthyl sulphate were two commonly used substrates in this search for arylsulphatases in different organisms and tissues. The chromogenic substrates; p-acetylphenyl sulphate and phenolphthalein disulphate (Barber *et al*, 1951; Dodgson *et al*, 1954) have also been used in the past although their use has fallen off because they have been found not to be hydrolysed by all known arylsulphatases. The same problem is found with the original substrate; indoxyl sulphate, which, along with the difficulty in obtaining it, has led to the very limited use of this compound as a substrate. It is thus always advisable to use more than one substrate when testing for sulphatases. Nitrocatechol sulphate and p-nitrophenyl sulphate are the most commonly employed although problems can arise with the latter if crude extracts are being assayed (Dodgson and Spencer, 1957). This results from adsorption of the liberated phenol to the protein so that when the protein is precipitated much of the product which is to be measured is lost. This is not a problem with purified enzyme preparations as very little protein is present and there is no need to remove it from the solution before measuring the absorbance. It was also demonstrated by Dodgson and Spencer (1953a) that the reduction of the nitro group of p-nitrophenol to form p-aminophenol by liver homogenates can lead to large losses of p-nitrophenol and therefore erroneous results. A similar reduction of 4-nitrocatechol may also occur

but does not appear to be as prevalent.

The relative ease of assaying these arylsulphatases with these substrates has led to a more extensive study of them than has been carried out with the other sulphatases mentioned above.

A. Mammalian Enzymes

In the early 1950's work began by Dodgson and Spencer and by Roy to purify some of the aryl sulphatases and to characterize them kinetically. This work led to the discovery in mammalian tissue of several different types of aryl sulphatase which were distinguishable by their kinetics. The presence of these three individual sulphatases, termed A, B and C, was confirmed when the enzymes A and B were purified and different physical properties observed with each. The three enzymes seem to be distributed throughout the mammal, all being present to some extent in the tissues studied. They are located within different parts of individual cells, however, sulphatases A and B being lysosomal in origin while sulphatase C is found in the microsomes.

There appears to be a difference in the location of sulphatase A and sulphatase B within the lysosomes as sulphatase A can be extracted from ox liver 'lysosomes' with water alone while sulphatase B must be extracted with 0.15 M KCl, treatment with butan-1-ol, or by lowering the pH (Roy, 1960). This difference could reflect a heterogeneous population of lysosomes or the two enzymes being present in different component structures of single lysosomes. The heterogeneity of the lysosomes in rat

liver has been demonstrated both through the comparison of the lysosomes of different cells, e.g. hepatocytes and Kupffer cells, which are quantitatively different in their general enzyme content (Hook *et al*, 1973; Arborgh *et al*, 1973; Munthe-Kaas *et al*, 1976) and by studies of the lysosomes within a single population of cells. Pertoft *et al* (1978) separated a high-density and low-density population of lysosomal particles from rat liver parenchymal cells on iso-osmotic self-generating gradients but suggested that the two groups reflected different stages in a common metabolic route for the lysosomes. Evidence indicated that as the lysosomes aged they passed from the low to the high-density population. It was also demonstrated that an arylsulphatase in the high-density lysosomes had a higher isoelectric point (sulphatase B?) than the enzyme associated with the low-density lysosomes (sulphatase A?). This observation was used as further evidence of the "ageing" of the lysosomes as Goldstone and Koenig (1974b) had previously shown that the lysosomal acid hydrolases, including sulphatases, became more basic as they aged due to the loss of N-acetyl-neuraminic acid residues to form isoenzymes. The arylsulphatase mentioned by Pertoft was not studied in sufficient detail to determine whether it was an A and/or B enzyme and therefore whether either enzyme was more abundant in one population of lysosomes. The possibility that the B enzyme, which is the more basic of the two, is derived from the A form will be discussed below.

Both the A and B enzymes are inhibited by sulphate, although the inhibition with sulphatase A functioning as

an arylsulphatase is competitive while that with sulphatase B is non-competitive, indicating a difference between the mechanisms of the two. Also, sulphatase B does not exhibit the anomalous kinetics of sulphatase A.

When the enzymes were purified it was found that they also differed in their charge and molecular weight. Sulphatase A is larger and has a lower isoelectric point than sulphatase B. A comparison of the physical and kinetic constants determined for the ox liver enzymes is given in Table 3.

Several people have sought to find a relationship between the A and B enzymes but evidence to date indicates that they are completely independent. Goldstone *et al* (1971) suggested that removal of the sialyl residues of sulphatase A would convert it to sulphatase B but the work of Graham and Roy (1973) with the ox liver enzyme showed this not to be the case. Desialylated sulphatase A and sulphatase B differed in molecular weight, charge and enzyme activity. It was later suggested by Goldstone and Koenig (1973) that sulphatase A was formed from sulphatase B during movement through the endoplasmic reticulum and Golgi apparatus to the lysosomes and that sulphatase B could be reformed through intracellular autolysis (Goldstone and Koenig, 1974a; Sanghavi and Koenig, 1976). Proof of this hypothesis has not been obtained and because of the different specificities and kinetic parameters of the enzymes it seems questionable. It has also been demonstrated that there is no immunological cross-reactivity between the two enzymes (Shapiro

TABLE 3

PHYSICAL PROPERTIES OF OX LIVER SULPHATASES A AND B

	Sulphatase A		Sulphatase B
	pH 7.5	pH 5.0	pH 7.5
Molecular Weight (equilibrium centrifugation)	107 000	411 000	56 000
Mobility $\times 10^5$ (cm^2 sec^{-1} V^{-1})	-9.9	-5.3	
Net Charge	-30	-9	
$E^{1\%}_{280\ nm}$	7.0	7.0	14
Isoelectric Point (pI)	3.4		8.3
$D^* \times 10^7$ (cm^2 sec^{-1})	4.9	3.2	6.6

	K_m (mM)	V^+	K_m (mM)
p-nitrophenyl sulphate	223	1.0	10.8
nitrocatechol suphate	1.0	0.84	1.8
sulphate	$K_i = 0.2$ mM		$K_i = 1.2$ mM

$^+$relative to p-nitrophenyl sulphate

and Nadler, 1975; Rhodes *et al*, 1976) giving no evidence for the existence of common subunits.

The sulphatases B have been shown to exist as different isoenzymes. Allen and Roy (1968) separated two isoenzymes (Bα and Bβ) from ox liver by chromatography on CM-Sephadex which were indistinguishable kinetically and by their molecular weights but presumably differed in their charge. Isoelectric focusing of each of these enzymes produced further separation into fractions called $B\alpha_1$, $B\alpha_2$, $B\beta_1$ and $B\beta_2$. Similar results were found by Bleszynski (1967) and Blesznyski and Roy (1973) for the ox brain enzyme. In this case three isoenzymes were found which could be further separated into α, β and γ fractions. Three isoenzymes of sulphatase B extracted from human placenta have also been found which differ in their molecular weight and substrate affinity (Gniot-Szulzycka, 1972).

A phosphorylated brain-specific arylsulphatase, termed Bm, has been found in human and monkey brain. This enzyme can be separated from sulphatase B by DEAE-cellulose chromatography (Stevens *et al*, 1977; Lakshmi and Balasubramanian, 1980). It loses 85% of its activity when treated with neuraminidase and dephosphorylation does not convert it into sulphatase B. A phosphorylated form of sheep brain sulphatase A has also been observed by Das and Bishayee (1980) and shown to be membrane bound while the non-phosphorylated form is soluble.

Sulphatase C is an insoluble enzyme found in the microsomes. The rat liver enzyme has been solubilized

(Dodgson *et al*, 1957) but pure forms of sulphatase C have not been obtained and its kinetics have been studied using crude extracts only. It has a pH optimum of approximately 7.0-8.5 for the hydrolysis of p-nitrophenyl sulphate and, unlike the A and B enzymes, is not inhibited by sulphate. The anomalous kinetics observed with sulphatase A are not found with sulphatase C.

B. Other Arylsulphatases

Arylsulphatases have also been found in plants, microorganisms and invertebrates. Two general types have been defined from the observations made with these enzymes and the mammalian ones.

Type I

A type I arylsulphatase was originally defined as an enzyme which was not inhibited by sulphate or phosphate but was inhibited by cyanide and fluoride (Dodgson and Spencer, 1957). The definition has since been reduced to an enzyme which is not inhibited by sulphate as the response to all of the above-mentioned compounds was not as universal as first thought. For example, the enzyme isolated from *Aerobacter aerogenes* is not inhibited by sulphate but is inhibited by phosphate, cyanide and fluoride (Fowler and Rammler, 1964). Type I sulphatases have been obtained from both mammalian and microbial sources. An example of the former type is the sulphatase C of liver microsomes which has not yet been purified and its kinetics have been studied using crude extracts. Interpretation of such data is difficult and it will only be properly characterized when obtained in a

homogeneous form. Sulphatase C does appear, however, to have an alkaline optimum pH and shows greater activity with p-nitrophenyl sulphate and p-acetylphenyl sulphate than with nitrocatechol sulphate.

Aspergillus oryzae, *Aerobacter aerogenes* and *Alcaligenes metalcaligenes* have all been used as sources of type I arylsulphatase. The *A.oryzae* enzyme has been separated into three isoenzymes; I, II and III, by electrophoresis. Work done with the II isoenzyme suggests that histidine and tyrosine residues are involved in the active site. As will be discussed in Chapter 7, these residues also seem to be essential to the type II arylsulphatases found in mammalian tissue. The *A.oryzae* enzyme is inhibited by sulphite, fluoride and phosphate but not by sulphate, chloride or cyanide. The enzymes from *A.aerogenes* and *A.metalcaligenes* are anticompetitively inhibited by cyanide.

Table 4 gives some of the properties of these enzymes. The anomalous kinetics described for sulphatase A are not observed with any type I enzymes.

Type II

Again, the original definition of a type II enzyme was one which was inhibited by sulphate and phosphate but not by cyanide or fluoride. The criteria was then reduced to sulphate inhibition. These enzymes are common in animal tissue but there is only one case where it has been found in a microorganism. Dodgson (1959) isolated an enzyme from *Proteus vulgaris* which would catalyze the removal of sulphate from p-nitrophenyl sulphate and nitrocatechol

TABLE 4

TYPE I ARYLSULPHATASES

	Source					
	A. oryzae		*A. metalcaligenes*		ox liver	
	K_m (mM)	V^+	K_m (mM)	V	K_m (mM)	V
p-nitrophenyl sulphate	0.17 (pH 6.2)	1.0	4.8 (pH 8.8)	1.0	2.0 (pH 8.0)	1.0
p-acetylphenyl sulphate			0.9 (pH 8.7)	1.0		
nitrocatechol sulphate	0.35 (pH 5.9)	0.67	1.7 (pH 7.8)	0.14	8.0 (pH 7.5)	0.5

$^+$relative to p-nitrophenyl sulphate

sulphate and was inhibited by sulphate. This enzyme did not exhibit the anomalous kinetics mentioned above nor did the type II enzymes isolated from the digestive glands of the molluscs *Helix pomatia* and *Patella vulgata*. In contrast to the type I enzymes, the type II have acid optimum pH's and early work suggested that they had greater activity towards nitrocatechol sulphate than p-nitrophenyl sulphate. As assay methods have improved and initial velocities calculated this has been shown not to necessarily be the case. For example, a V_o of 234 µmole min^{-1} mg^{-1} with nitrocatechol sulphate and a V_o of 277 µmole min^{-1} mg^{-1} with p-nitrophenyl sulphate was measured with the sulphatase A of ox liver. With this enzyme at least, the apparently lower activity observed with p-nitrophenyl sulphate was due to the much higher Km (i.e. Km = 1.0 mM nitrocatechol sulphate, Km = 223 mM p-nitrophenyl sulphate) meaning that the substrate concentration used in assays was a small fraction of the Km value.

1.22 Metabolic Role

The wide distribution of arylsulphatases has made it difficult to postulate their metabolic roles. In microorganisms where sulphur metabolism is quite well understood, they are believed to be involved in the conversion of inorganic forms of sulphur to sulphur containing amino acids. Evidence for this was observed in *Aerobacter aerogenes* (Rammler *et al*, 1964; Harada and Spencer, 1964) where the synthesis of arylsulphatase was repressed by cysteine and other compounds believed to occur on the metabolic pathway from sulphate to cysteine.

In molluscs they are thought to play a role in the production of coloured quinoid compounds which probably serve a protective function. These pigments are formed by the hydrolysis of sulphate ester precursors followed by their oxidation. As the enzymes are also present in the digestive juices of these molluscs they may be important to digestion as well.

In mammalian systems their role was less clear and the task of proposing one was made more onerous by the fact that for many years their only known substrates were not thought to have a physiological function. The sulphation of phenols was believed to be a method of detoxification and it seemed unlikely that they would hydrolyse such compounds. In 1960 Austin observed that the disease metachromatic leukodystrophy was characterized by an accumulation of cerebroside sulphates in myelin, kidney and other tissues, the first of which leads to brain damage. He later found a correlation between the disease and low arylsulphatase activity in the brain, liver and kidney (Austin *et al*, 1963; Austin *et al*, 1965). Mehl and Jatzkewitz (1964, 1968) subsequently demonstrated that sulphatase A from pig kidney was capable of hydrolysing cerebroside sulphate and that the two activities copurified to homogeneity. This indicated that a single enzyme was responsible for both activities and therefore that the two enzymes which had previously been thought to be non-identical were actually the same. A physiological role could then be assigned to this enzyme and evidence has been obtained to show that sulphatase A

is responsible for hydrolysing cerebroside sulphate *in vivo*. It has been demonstrated that cultured fibroblasts from metachromatic leukodystrophy patients are able to incorporate sulphatase A from their growth medium and subsequently are indistinguishable from normal fibroblasts in their hydrolysis of cerebroside sulphate (Porter *et al*, 1971).

Many other deficiency diseases have been observed in man which are caused by a lack of sulphatase(s). Multiple sulphatase deficiency disease results from lowered levels of many sulphatases, including sulphatases A, B and C and is characterized by the accumulation of glycosaminoglycans, cholesterol sulphate and cerebroside sulphate. This suggests that glycosaminoglycans are natural substrates for sulphatase B but the raised levels observed with Hurler, Hunter and Sanfilippo B syndromes (all mucopolysaccharidoses) make this questionable. Sulphatase B activity has been demonstrated to be deficient in the liver, kidney, spleen, brain and cultured fibroblasts of patients suffering from Maroteaux-Lamy syndrome. The enzyme may therefore be important as an O-sulphatase in the catabolic pathways of sulphated mucopolysaccharide metabolism (Stumpf *et al*, 1973). It may specifically be responsible for the desulphation of the N-acetylgalactosamine 4-sulphate found in dermatan sulphate, a compound known to accumulate in the fibroblasts and other tissues of these patients (O'Brien *et al*, 1974). On the evidence of the increase in the ratio of sulphatase B to sulphatase A in cartilage from patients with degenerative joint disease, Schwartz *et al* (1974) suggested that oligosaccharides of chondroitin sulphate and keratan sulphate may also be

physiological substrates for the B enzyme. An instance of a correlation between a disease state and low levels of a sulphatase other than an arylsulphatase is Hunters Syndrome. The symptoms of a patient suffering from this disease, which results from a deficiency of sulpho-L-iduronate sulphatase, were shown to decrease after exposure to the normal enzyme (Dean *et al*, 1975). In this case a skin allograft of normal tissue proved effective therapy until it was rejected. This enzyme is thought to be distinct from sulphatase B because no activity was observed with the latter using sulpho-L-iduronic acid as substrate.

Yet another sulphatase has recently been found in human urine which is specific for the 3-O-sulphate ester of sulphaminoglucopyranoside. The enzyme has not been fully characterized but it was shown that the hydrolysis of this ester is dependent on the amino group of the above compound also being sulphated, has an optimum pH of 6.3 and is inhibited by inorganic sulphate and phosphate. It is suggested that such a 3-O-sulphate ester may exist in heparan sulphate or heparin and that this enzyme may have a role in its physiological activity (Leder, 1980).

1.3 OX LIVER SULPHATASE A

The characteristics of the sulphatase A isolated from ox liver will now be considered in greater detail. It should be noted that the pattern which emerges with this enzyme is similar to that found with sulphatase A purified from other sources such as rabbit liver (Lee and Van Etten, 1975a), rabbit testes (Yang and Srivastava, 1976), pig kidney (Mehl and Jatzkewitz,1964), beef brain

(Bleszynski *et al*, 1969), human urine (Stevens *et al*, 1975), human kidneys (Stinshoff, 1972) and human liver (Draper *et al*, 1976). All of these enzymes exhibit the characteristic anomalous kinetics of sulphatase A and respond to effectors in the same way. They are also quite similar in their physical properties such as their subunit structure, polymerization pattern and isoelectric point.

1.31 Anomalous Kinetics

The sulphatase enzymes of ox liver were first detected by Roy in 1953. Sulphatase A was separated from sulphatase B by fractional precipitation with acetone and, as nitrocatechol sulphate was used as substrate, its anomalous kinetics were obvious in comparison to the more standard kinetics of the B enzyme. The enzyme had not been purified at this stage and its activity was being measured by the amount of product released in an one hour incubation. The anomaly therefore appeared in the non-linear relationship between enzyme concentration and activity. Once the time course had been followed more closely (Dodgson and Spencer, 1956b, using the human enzyme) it was evident that the enzyme was inactivating and, with the higher enzyme concentrations, this was followed by a reactivation. It was thus a difference in the degree of reactivation found with the various concentrations of enzyme that produced the non-linear relationship. Dodgson and Spencer (1956b) demonstrated that the inorganic sulphate produced from the substrate during the reaction was the major factor effecting the kinetics but that nitrocatechol, the concentration of substrate, pH and

temperature also had effects.

The bottom line in Figure 1 is the progress curve produced by ox liver sulphatase A, at a concentration of 0.1 μg ml^{-1}, hydrolysing 3 mM nitrocatechol sulphate at pH 5.6, I=0.1. The rate of hydrolysis has dropped off radically in the first 10 minutes and remains low during the remaining 50 minutes, indicating that the enzyme has been inactivated. In the curve above the concentration of enzyme is increased to 0.4 μg ml^{-1}. Again the decrease in hydrolysis is observed but a spontaneous reactivation occurs if the reaction is allowed to proceed. This reactivation was shown to be caused by the accumulation of sulphate, one of the products of the reaction catalyzed, by Baum and Dodgson (1958). By adding $BaCl_2$ to the reaction after activation had occurred they produced an immediate loss of activity which could be reversed if an excess of sulphate was added to the reaction mixture. The other product, nitrocatechol, is also capable of reactivating the modified enzyme but it is much less effective than sulphate and would be overshadowed by the latter under most conditions. The curve can be conveniently divided into three sections. In the first the enzyme is being inactivated, in the second it has lost most of its activity, and in the third it is being reactivated. At the lower enzyme concentration insufficient sulphate was produced in the initial stage to cause reactivation later on. Figure 2 shows the curve obtained when K_2SO_4 is added to the reaction mixture containing 0.1 μg ml^{-1} sulphatase A after the rate of

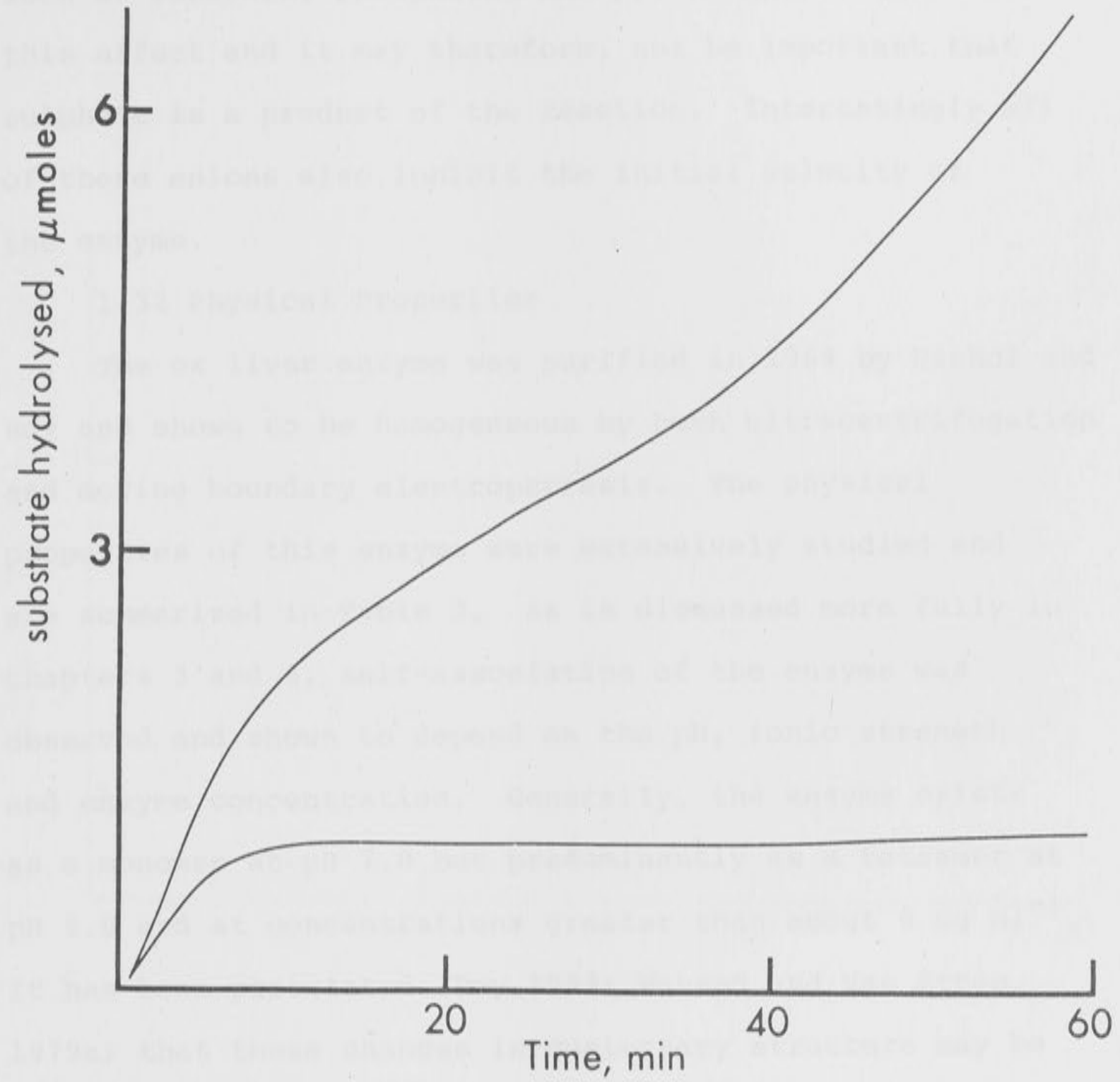


Figure 1 pH-stat recordings showing the effect of enzyme concentration on the time course of the reaction. Native ox liver sulphatase A has been added to 3 mM nitrocatechol sulphate, pH 5.6, 0.1 M KCl to initiate the reaction. The enzyme concentration is 0.1 μg ml^{-1} in the lower curve and 0.4 μg ml^{-1} in the upper curve.

hydrolysis has decreased. As can be seen, similar reactivation can be induced in this way. Other anions, such as fluoride, phosphate, and pyrophosphate also have this effect and it may therefore, not be important that sulphate is a product of the reaction. Interestingly all of these anions also inhibit the initial velocity of the enzyme.

1.32 Physical Properties

The ox liver enzyme was purified in 1964 by Nichol and Roy and shown to be homogeneous by both ultracentrifugation and moving boundary electrophoresis. The physical properties of this enzyme were extensively studied and are summarized in Table 3. As is discussed more fully in Chapters 3 and 6, self-association of the enzyme was observed and shown to depend on the pH, ionic strength and enzyme concentration. Generally, the enzyme exists as a monomer at pH 7.0 but predominantly as a tetramer at pH 5.0 and at concentrations greater than about 5 $\mu g\ ml^{-1}$. It has been postulated (Roy,1953; Waheed and Van Etten, 1979a) that these changes in quaternary structure may be involved in the anomalous kinetics. Jerfy *et al* (1976) showed that the monomer followed Michaelis kinetics but the tetramer did not if assayed with nitrocatechol sulphate and 0.1 M NaCl. Negative cooperativity was observed between the monomer units. These results might suggest that, as the two polymeric states of the enzyme differed kinetically, the association-dissociation reaction may be involved in the anomalous kinetics. Further measurements made under the conditions used for determining cerebroside

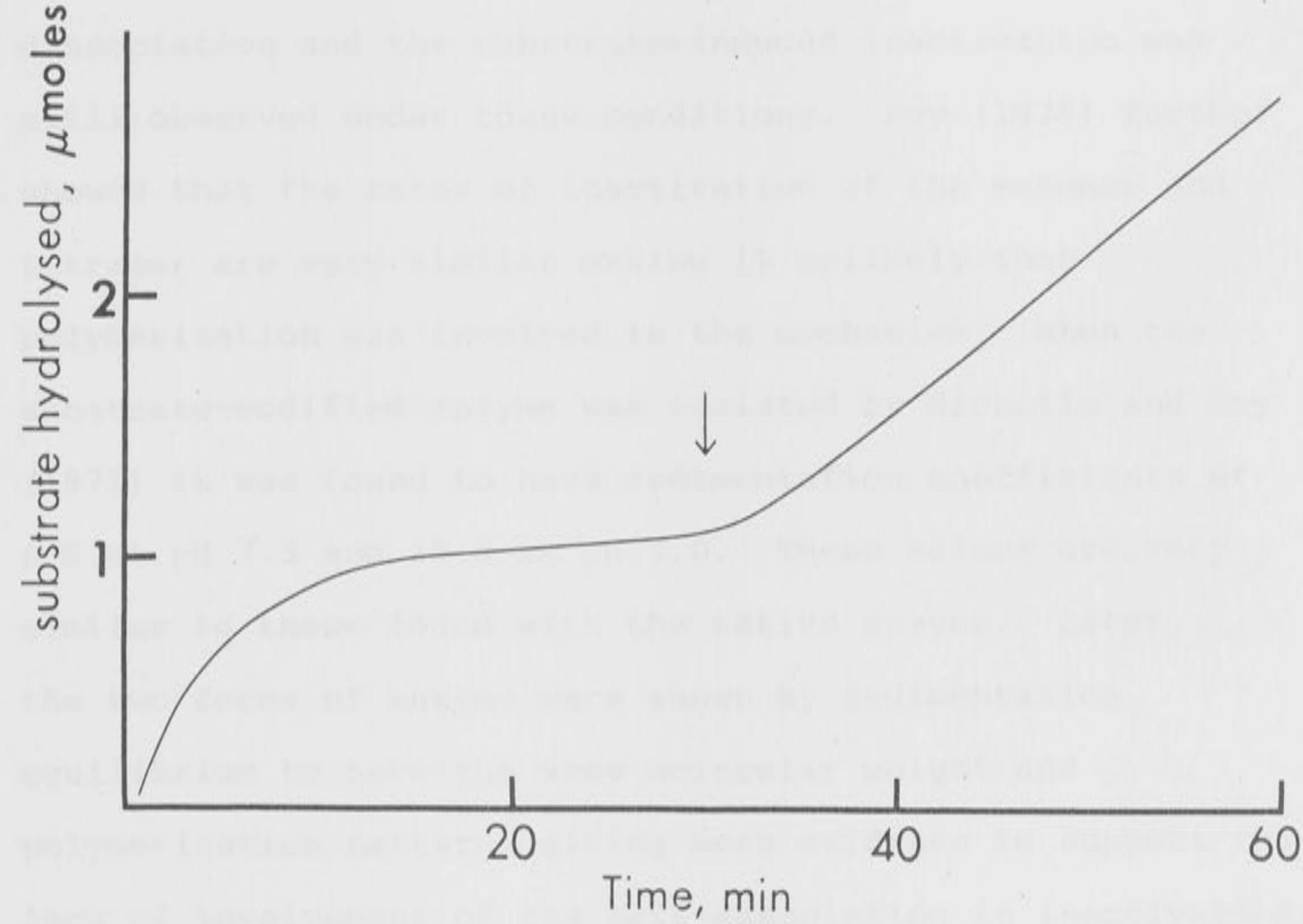


Figure 2 pH-stat recording of the hydrolysis of 3 mM nitrocatechol sulphate, pH 5.6, I = 0.1, by 0.1 μg ml^{-1} native sulphatase A. Thirty μmoles of sulphate have been added at 30 minutes.

sulphatase activity, i.e. 2 mM sodium taurodeoxycholate and 0.035 M $MnCl_2$, suggest that this is not the case. In this reaction mixture the tetramer did follow Michaelis kinetics and therefore the catalytic activities of the monomer and tetramer are similar under these conditions. The change in kinetics was shown not to result from dissociation and the substrate-induced inactivation was still observed under these conditions. Roy (1978) further showed that the rates of inactivation of the monomer and tetramer are very similar making it unlikely that polymerization was involved in the mechanism. When the substrate-modified enzyme was isolated by Nicholls and Roy (1971) it was found to have sedimentation coefficients of 6 S at pH 7.5 and 14 S at pH 5.0. These values are very similar to those found with the native enzyme. Later, the two forms of enzyme were shown by sedimentation equilibrium to have the same molecular weight and polymerization pattern, giving more evidence to support the lack of involvement of the self-association in inactivation.

Dissociation of the monomer into two or possibly four subunits has been observed on the addition of denaturants such as urea and SDS. Although it has never been suggested that this dissociation was involved in the anomalous kinetics, interactions between the subunits may be. This is discussed more fully in Chapter 3 and therefore the details of the dissociation will not be given here.

1.33 Amino Acid Composition

The amino acid composition of the enzyme was determined

by Nichol and Roy (1965) and it was shown to be a glycoprotein. Further measurements by Graham and Roy (1973) showed it to contain 2% sialic acid and probably 8 carbohydrate side chains. The latter is presumed because neuraminidase treatment removed all 8 sialic acid residues and only those in terminal positions would be expected to be effected. The enzyme monomer contains 18 glucosamine residues, 14 mannose residues, 8 galactose residues and 8 sialic acid residues.

Little comment need be made regarding the amino acid composition other than pointing out the high concentration of proline (90 residues). This should be considered when analyzing the secondary structure of the molecule as proline is thought to disrupt the helical structure of proteins (Blout and Fasman, 1958)

1.34 Kinetics

Table 5 gives some of the kinetic constants which have been determined for ox liver sulphatase A. The large number of sulphate esters which have been used as substrate for this enzyme is evident from the Table. These compounds will be discussed below along with the methods of assay they are employed in. The maximum velocity found with a number of these substances is similar suggesting that the rate-limiting step of the reaction with these particular substrates is the liberation of sulphate (Roy, 1980). Many of the substrates used, however, had a much lower value of V which suggests that in these cases the rate-limiting step must occur at an earlier stage. As little is known of the mechanism of catalysis it is difficult to explain

TABLE 5

KINETIC CONSTANTS FOR OX LIVER SULPHATASE A

Substrate	Km (mM)
nitrocatechol sulphate	1.0
nitroquinol sulphate	5.9
2-nitropyridyl 3-sulphate	5.9
2-nitrophenyl sulphate	12.3
ascorbate 2-sulphate	24.3
4-methylumbelliferone sulphate	41.0
2-napthyl sulphate	40.6
3-nitrophenyl sulphate	104
4-nitrophenyl sulphate	223

Inhibitors	Ki
sulphate	0.2 mm
sulphite	0.2 μm

this difference. Sulphate, sulphite and phosphate all inhibit the native enzyme competitively when aryl sulphates are used as substrates. This is consistent with sulphate being the last released product of a uni-bi reaction. It has been noted that not only the reduction of the initial velocity is caused by the presence of these anions but also a reduction in the rate of the substrate-induced inactivation. This demonstrates the association between the catalytic reaction and the inactivation process which is apparent experimentally if not theoretically. Inhibition has also been observed with metal ions such as Cu^{++} and Hg^{++} which is probably due to their reaction with sulfhydryl groups.

Sulphate, phosphate, pyrophosphate and sulphite all inhibit the initial velocity and activate the substrate-modified enzyme. As can be seen in the curve in Figure 2, where activation was initiated by the addition of K_2SO_4, the response to the activator is not immediate. A time lag, which has been shown to depend on the substrate used, its concentration and the concentration of activator, is observed before the maximum rate of hydrolysis is reached. Once this velocity is attained the reaction becomes linear.

It should also be noted that if the concentration of substrate or activator is increased above an optimal value the amount of activity which is restored during this third stage is reduced. That is, both substrate and activator inhibit the activation if their concentrations are raised. As will be seen in Chapter 5 the point at which inhibition becomes evident is interdependent on the substrate used,

its concentration and the concentration of activator added. It has been suggested that this is due to the presence of two binding sites on the substrate-modified enzyme each of which can bind either substrate or activator. Figure 3 is a diagram showing the reaction mechanism originally proposed by Baum and Dodgson (1958) and simplified by Nicholls and Roy (1971) for sulphatase A acting as an arylsulphatase. According to this scheme the enzyme-substrate complex either dissociates to form products and native enzyme or is converted into a second form of the enzyme, F, which then binds a second substrate molecule to form a complex FS_2. It was proposed that it was this complex that was the inactive form of the enzyme. Sulphate was thought to cause activation through the formation from FS_2 of FIS which was believed to be an active form of the enzyme. This scheme will be discussed in greater detail in Chapter 8 in light of the additional information provided by this present study of the ox liver enzyme and by studies which have recently been carried out with enzymes from other sources.

1.35 Cerebroside Sulphatase Activity

The kinetic behaviour of sulphatase A with cerebroside sulphate as substrate differs from that observed with the sulphate esters mentioned above. With cerebroside sulphate normally either an activator protein or manganous ions and bile salts, such as sodium taurodeoxycholate, are required for activity. Mehl and Jatzkewitz (1964) first observed that this protein, called the "complementary fraction" was required for cerebroside sulphatase activity. This factor

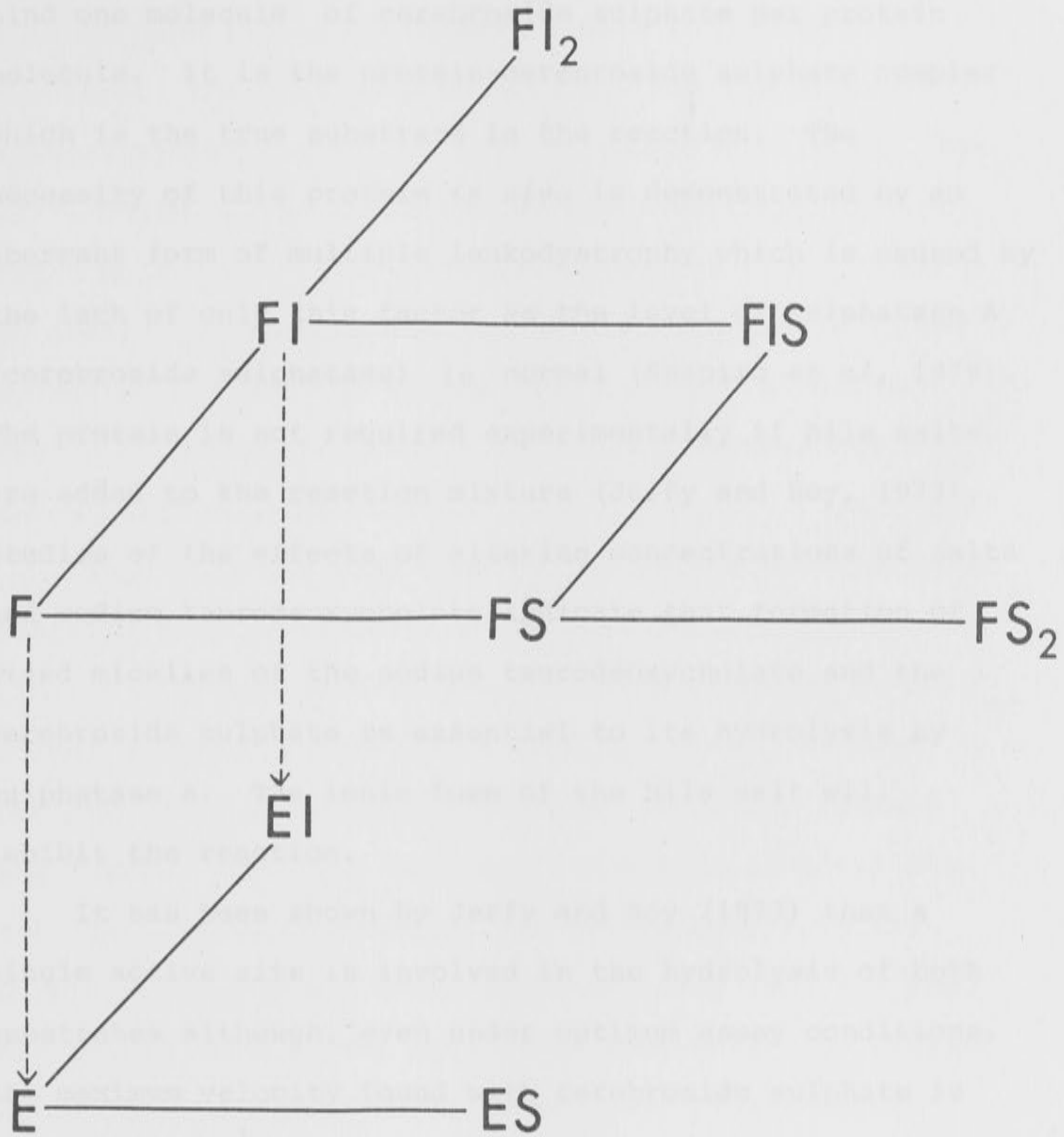


Figure 3 Reaction scheme proposed by Nicholls and Roy (1971) for sulphatase A acting as an arylsulphatase. The native enzyme is represented as E, the substrate-modified enzyme as F, the substrate as S and the sulphate (or other anion) as I.

was later shown to be a lysosomal protein which when isolated (Fisher and Jatzkewitz,1977, 1978) was shown to bind one molecule of cerebroside sulphate per protein molecule. It is the protein-cerebroside sulphate complex which is the true substrate in the reaction. The necessity of this protein *in vivo* is demonstrated by an aberrant form of multiple leukodystrophy which is caused by the lack of only this factor as the level of sulphatase A (cerebroside sulphatase) is normal (Shapiro *et al*, 1979). The protein is not required experimentally if bile salts are added to the reaction mixture (Jerfy and Roy, 1973). Studies of the effects of altering concentrations of salts and sodium taurodeoxycholate indicate that formation of mixed micelles of the sodium taurodeoxycholate and the cerebroside sulphate is essential to its hydrolysis by sulphatase A. The ionic form of the bile salt will inhibit the reaction.

It has been shown by Jerfy and Roy (1973) that a single active site is involved in the hydrolysis of both substrates although, even under optimum assay conditions, the maximum velocity found with cerebroside sulphate is approximately $^{1}/_{16}$ of that found with nitrocatechol sulphate. In contrast to arylsulphatase activity, cerebroside sulphatase activity is non-competitively inhibited by sulphate and activated rather that inhibited by hydroxylamine-HCl. This suggests that a different catalytic mechanism is involved with this substrate in which sulphate is not the last released product.

It was suggested (Jerfy and Roy, 1973) on the basis of kinetic data that the order of release was cerebroside, sulphate and lastly taurodeoxycholate in a uni-ter reaction. There is also no evidence for the production of substrate-modified enzyme with cerebroside sulphate (Roy, 1979) which may be explained by the involvement of the third molecule. It is also possible that the difference in mechanism might be due to the difference in size and hydrophobicity of the cerebroside molecule allowing a region of non-polar binding to develop between the cerebroside and the enzyme.

Some work has been done on the sulphatase A enzyme isolated from metachromatic leukodystrophy patients and kinetic differences have been observed compared to the normal enzyme. Sulphatase A from the urine of these patients did not exhibit substrate inhibition (Stumpf and Austin, 1971) and although the enzyme isolated from the liver would cross-react immunologically with the normal enzyme it had no residual activity (Shapira and Nadler, 1975). It was also noted that while the binding of the antibody increased the activity of the normal enzyme it had no effect on the mutant enzyme. The physical or chemical difference between these two enzymes has not been determined; the investigation being hindered by the small amounts of mutant enzyme attainable.

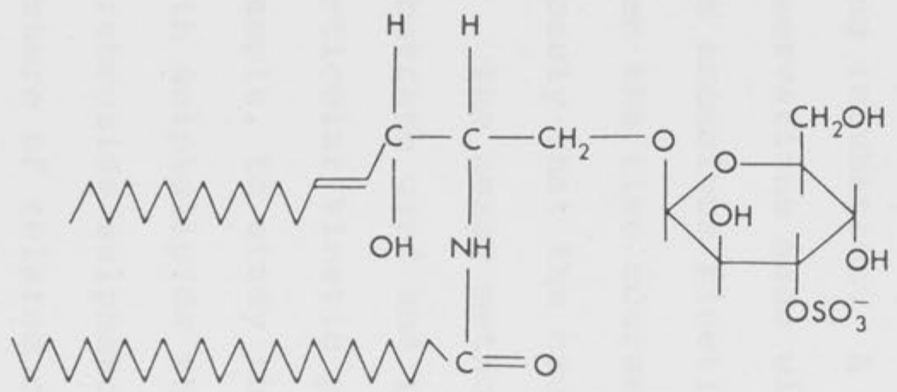
1.36 Other Substrates

It has further been shown that as well as cerebroside sulphate, sulphoglycerogalactolipids where the ester sulphate group is present at position 3 of a D-galactose

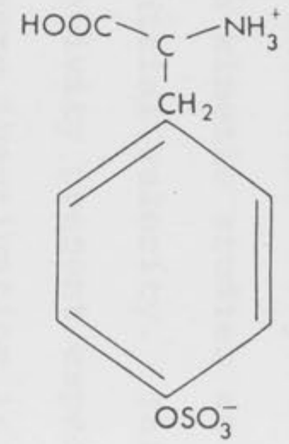
residue are natural substrates for mammalian sulphatase A. For example, seminolipid (Yamato *et al*, 1974; Fluharty *et al*, 1974) and psychosine sulphate (Eto *et al*, 1974) are both hydrolyzed if a bile salt is present in the reaction mixture as is the sphingolipid lactosyl sulphatide (Harzer and Benz, 1974). Simpler carbohydrate compounds such as glucose-3-sulphate, can also act as substrates. This reaction is competitively inhibited by sulphate and the formation of the substrate-modified enzyme is observed (Roy, 1980). Glucose 6-sulphate, galactose 2-sulphate and galactose 6-sulphate are also hydrolyzed as is adenosine 3'5'-monophosphate (Egami and Uchida, 1978), but at slower rates.

Other naturally occurring compounds have been found which are hydrolysed by sulphatase A although the metabolic role, if any, of these reactions is not known. Roy (1975) showed that ascorbate 2-sulphate was hydrolyzed by ox liver sulphatase A. At pH 4.8, the optimum pH for this reaction, a specific activity of 90 μmole min^{-1} mg^{-1} was detected. Although the kinetics were not Michaelis at this pH they were at pH 5.6 where the K_m is 21 mM. It was also noted that the anomalous kinetics of the enzyme are evident in this reaction. Tyrosine O-sulphate is another substrate of possible physiological significance. It has a K_m of 35 mM and is hydrolyzed at a rate much slower than nitrocatechol sulphate but comparable to that observed with cerebroside sulphate.

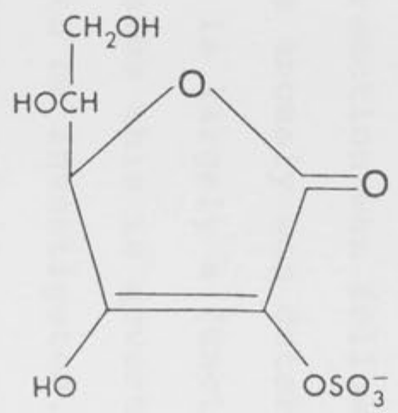
The structures of some of these compounds are shown in Figure 4.



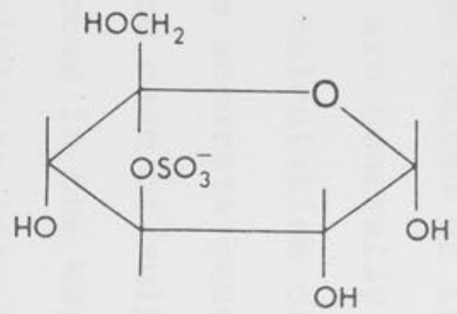
cerebroside sulphate



tyrosine O-sulphate



ascorbate 2-sulphate



glucose 3-sulphate

Figure 4 Some naturally occurring sulphate esters which are hydrolysed by sulphatase A.

1.4 ASSAY OF SULPHATASE ACTIVITY

Several methods have been developed for either determining the activity of a sulphatase A solution from the amount of product released in a given time (discontinuous assays) or following the time course of the reaction (continuous assays). The latter are generally preferable in kinetic studies as they allow calculation of the initial velocity. This is a more accurate gauge of the activity present especially in the case of sulphatase A where inactivation is known to occur in the early stages of the reaction. It is also possible to lose a great deal of kinetic information if the enzyme activity is measured only as the amount of product formed during a long incubation. A good example of this is the initial observations made with the ox liver sulphatase A regarding its anomalous kinetics. As described above it was only when the time course of the reaction was followed more closely that the cause of the anomaly was determined.

The assay method chosen is largely a function of the substrate used and in many cases this is governed by the particular kinetic property to be investigated. For example, to study the sulphatase activity of sulphatase A with sulpholipids the only substrate of practical use is cerebroside sulphate which is commercially available as a mixture of related compounds. If the activation of the substrate-modified sulphatase A is of interest then nitrocatechol sulphate is the most likely substrate to be used because the reaction reaches a steady state more rapidly with this substrate than with others.

1.41 Determination of Unhydrolysed Substrate

A method developed by Vlitos (1953) for measuring sodium 2-(2,4-dichlorophenoxy)ethyl sulphate in soil was first applied to the assay of sulphatase activity by Roy (1956). It was used to measure the activity of steroid sulphatases from the mollusc *Patella vulgata* and ox liver and involves determining the concentration of methylene blue complexes formed from the ester and methylene blue chloride in acidic solution. The complex is extracted into chloroform and its concentration calculated from the absorbance at 650 nm of this solution. The method is limited by the fact that in order to obtain a significant difference between the control (the initial concentration of substrate) and the assay (the final concentration of substrate) a large percentage of the substrate must be hydrolysed. The substrate concentration therefore cannot be assumed to remain constant throughout the assay.

A sensitive spectrophotometric assay for sulphatides has been developed by Kean (1968) and its use in determining sulphatase activity was suggested. In this case the concentration of a complex of the cationic dye Azure A and the anionic sulpholipid is determined.

1.42 Determination of Inorganic Sulphate

A method of determining sulphate by precipitation with benzidine was used by Dodgson and Spencer (1953b) in a study of sulphatases. Use of this method was limited by the fact that if less than 10 µg SO_4^{2-} was present it did not precipitate quantitatively. This meant very long incubation periods were required when crude extracts were being measured, the results from which are difficult to

interpret. Also, because benzidine is carcinogenic other less hazardous assay methods are preferable.

Dodgson (1961) later developed a turbidimetric method which was accurate over a total concentration range of 0-200 µg SO_4^{2-} if slight variations were used in the procedure. This method was found to be suitable for assaying the activity of a purified glycosulphatase and alkylsulphatases.

Ginsberg and Di Ferrante (1977) modified the rhodizonate procedure for measuring sulphate and applied it to the assay of sulphatase activity. A similar method has also been used by Waheed and Van Etten (1978). This method has a lower limit of sensitivity of 1 nmole SO_4^{2-}. It is a spectrophotometric procedure which measures the pink colour produced when the dye potassium rhodizonate forms a complex with Ba^{2+}. The colour will be reduced if the concentration of Ba^{2+} is decreased through the formation of $BaSO_4$.

This approach suffers from the fact that it cannot be used continuously and also it is not possible to study the activation of the substrate-modified enzyme as the activators would all interfere with the measurements by their reaction with Ba^{2+}.

1.43 Spectrophotometry

As the absorption spectra of many of the sulphate esters which are hydrolyzed by sulphatase A change dramatically when the sulphate group is removed, activity can be conveniently measured spectrophotometrically. These assays are also very sensitive due to the large

extinction coefficients of the resultant phenols and the progress of the reaction can be continuously monitered by recording the absorption of the solution at the λ_{max} of the phenol.

Nitrocatechol sulphate (potassium 2-hydroxy-5-nitrophenyl sulphate) is useful only in discontinuous assays where the reaction mixture is made alkaline and the absorbance read at 510 nm (ϵ = 12 600). At pH 5.6, the optimum pH for the hydrolysis of this substrate by sulphatase A, the absorption spectra of nitrocatechol sulphate and nitrocatechol are similar and therefore it cannot be used for continuous assays in this method. The relatively low solubility of this compound limits the concentration range over which it can be employed. It is difficult to obtain a solution of more than 0.05 M at room temperature.

p-Nitrophenyl sulphate can be used in either discontinuous or continuous assays. The optimum pH for its hydrolysis by sulphatase A is 6.1 where the molar extinction coefficient of nitrophenol at 400 nm is 3 064. This substrate is unstable and readily degrades into nitrophenol and $KHSO_4$ on standing so its purity must be ascertained before use. This is best accomplished by purifying the compound. Alternatively kinetic equations were derived by Lee and Van Etten (1975a) for hydrolysis of a substrate contaminated with an inhibitor and these equations were used when p-nitrophenyl sulphate was substrate.

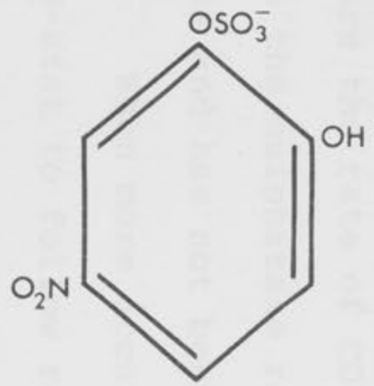
Nitroquinol sulphate (4-hydroxy-2-nitrophenyl sulphate) can also be used in continuous assays (Jeffrey and Roy,

1977). At pH 5.5, the optimum pH for hydrolysis, the absorption of nitroquinol and nitroquinol sulphate are sufficiently different ($\Delta\varepsilon$ = 2860 at 400 nm) for the reaction to be followed. This compound is more stable than p-nitrophenyl sulphate and can be dissolved in water to a concentration of 0.25 M at room temperature. The structure of these substrates is shown in Figure 5.

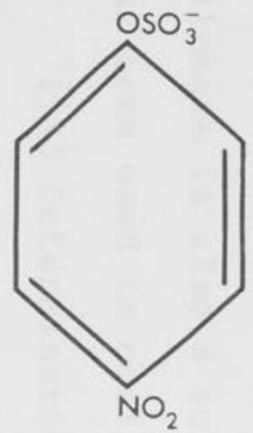
Dodgson and Spencer (1957) mentioned the use of p-acetylphenyl sulphate and phenolphthalein disulphate as substrates in spectrophotometric assays. They are not used as commonly as the above compounds, however, as sulphatases have been found which show no activity with these substrates. Specific examples of this are the enzymes from many strains of *E.coli* and *Aerobacter aerogenes* which hydrolyze p-nitrophenyl sulphate but not phenolphthalein disulphate (Harada *et al*, 1954). p-Acetylphenyl sulphate is useful for the assay of sulphatase C activity, however, as it can be hydrolysed by this enzyme under conditions where the activity of sulphatases A and B are negligible (Milsom *et al*, 1972) and phenolphthalein disulphate as a substrate for bacterial sulphatases (Whitehead *et al*, 1952;Dodgson *et al*, 1954).

1.44 Fluorometry

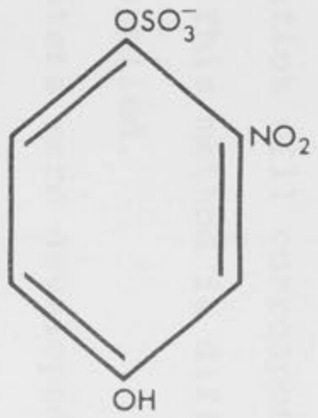
Mead *et al* (1955) showed that 4-methylumbelliferone sulphate could be accurately determined fluorometrically and suggested that it might be a useful substrate for arylsulphatases. Later Sherman and Stanfield (1967) and Guilbault and Hieserman (1969), using a highly purified



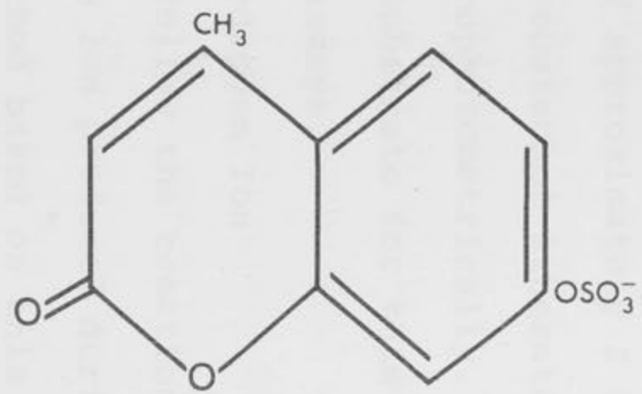
nitrocatechol sulphate
(2-hydroxy-5-nitrophenyl sulphate)



p-nitrophenyl sulphate



nitroquinol sulphate
(4-hydroxy-2-nitrophenyl sulphate)



4-methylumbelliferone sulphate

Figure 5 Some of the aryl sulphates commonly used as substrates for sulphatase A.

preparation of 4-methylumbelliferone sulphate to eliminate problems due to contamination with methylumbelliferone, demonstrated that assays for sulphatase activity could be carried out with much greater sensitivity fluorometrically. An increase in sensitivity of approximately 2 000 fold was found over measuring the nitrocatechol liberated from nitrocatechol sulphate spectrophotometrically. β-Naphthyl sulphate is also a suitable substrate for this assay and has been used in continuous assays.

1.45 Determination of Hydrogen Ion

It is also possible to follow the reaction by measuring the concentration of hydrogen ion produced during the hydrolysis. A manometric method based on this principle was used by Lowenstein and Young (1952) to measure the activity of an arylsulphatase from *Aspergillus oryzae*. If the reaction is carried out in a $NaHCO_3$-H_2CO_3 buffer one CO_2 molecule is produced for every H^+ released and therefore the rate of CO_2 accumulation will correspond directly to the sulphatase reaction. This method is difficult to use and has not been widely applied.

When more accurate pH meters were developed, use of a pH-stat to follow reactions involving the liberation of an acid and/or base became feasible. The pH-stat was first used for assaying arylsulphatases by Andersen (1959a), who measured the activity of the ox liver enzyme with nitrocatechol sulphate as substrate. This method, like the spectrophotometric method with p-nitrophenyl sulphate and nitroquinol sulphate, allows continuous measurement of the concentration of product released and therefore the

calculation of the initial velocity. It also has the advantage that any substrate can be employed whereas the spectrophotometric assay is limited to substrates which show a substantial change in absorption when hydrolysed and the fluorometric assay is confined to the use of either β-naphthyl sulphate or 4-methylumbelliferone sulphate. In practice problems can occur with some substances. For example, nitrocatechol sulphate, a commonly used substrate, has a pK of 6.5 and therefore a large buffering capacity at pH 5.6, the optimum pH for the hydrolysis of this substrate. As the method relies on the titration of H^+ produced during the reaction, the buffering by the unhydrolysed substrate and the product (nitrocatechol has a pK of 6.4) means the rate measured will be less than the true rate. As pointed out by Roy (1978) this will not affect the comparison of rates measured at the same substrate concentration and therefore the same buffer capacity but can lead to erroneous results if data obtained over a wide range of nitrocatechol sulphate concentration are compared. This is not a problem with the other substrates commonly used but will be if measuring a reaction at any pH where buffering occurs.

Using this method the effects of rising sulphate concentration and depletion of substrate can also be accurately eliminated. Andersen (1959a) demonstrated this by adding an equivalent of $BaCl_2$ to the base used as titrant, to precipitate the liberated sulphate, and an equivalent of substrate to maintain the original concentration.

A very sensitive protonometric method has also recently been applied to the assay of arylsulphatase activity by Roy (private communication). This method measures changes in the potential of a glass electrode caused by changes in H^+ concentration and will be discussed further in Section 2.12.

1.46 Radiochemical Assays

Porter *et al* (1972) developed a method of measuring cerebroside sulphate activity using ^{35}S labelled substrate. Extracts of cultured human fibroblasts were incubated with cerebroside [^{35}S]sulphate and then the inorganic sulphate was extracted and measured. Long periods of incubation were required, however, to get reliable data. This method was also used by Stevens *et al* (1975) for measuring sulphatase activity in human urine.

p-Nitrophenyl [^{35}S]sulphate has been used as a substrate by Kawiak *et al*, (1964) when determining the activity of whole cartilage. The released $^{35}SO_4^{2-}$ was precipitated with Ba^{2+} and located by autoradiography. Although this is a qualitative assay it was useful as he was able to show that the enzyme activity was randomly distributed through the chondrocytes.

Cerebroside sulphatase has recently been measured using [^{3}H]cerebroside sulphate (Poulos and Beckman, 1980). After incubation, the reaction mixture was shaken with a solution of galactose and chloroform, centrifuged, and the radioactivity of the upper phase determined. Stearlyl [1-^{14}C]sulphogalactosylsphingosine (Dubois *et al*, 1980) has also been used as a substrate for cerebroside

sulphatase. In this case the product was separated from unreacted substrate by extraction with n-butanol-diisopropyl ether and DEAE-cellulose chromatography. After the extraction the organic phase was dried and redissolved in chloroform methanol before applying to the column.

2. ANALYTICAL METHODS

2.1 DETERMINATION OF ARYLSULPHATASE ACTIVITY

2.11 pH-Stat Assay

The theory and use of the pH-stat is explained in detail by Jacobsen *et al* (1958). Some of the equations which were derived in that article are given below to point out the difficulties associated with these measurements.

In the case of the sulphatase A reaction the pH is maintained by titrating the acid produced in the reaction with NaOH. From the equation

$$pH = pK + \log b/a$$

where a and b are the concentrations of an acid A and its corresponding base B, the quantity of hydrogen ions which must be removed if the pH is to remain constant is

$$-n_{H^+} = A \times \frac{b}{a+b} = A \times \frac{10^{pH-pA}}{1+10^{pH-pA}}$$

where A is the total quantity of acid produced and pA is the pK value of the acid. This can also be expressed as

$$-n_{H^+} = B_R\beta_R = A\alpha \qquad (B_R = \text{base required})$$

where α and β are functions of (pH-pA) and will equal 1 for strong acids and bases respectively. As only one acid is produced in the sulphatase reaction the equation for the rate of uptake of hydrogen ions

$$- \frac{dN_{H^+}}{dt} = \sum_r \beta_r \frac{dB_r}{dt} - \sum_s \alpha_s \frac{dA_s}{dt}$$

for r bases and s acids, reduces to

$$- \frac{dN_{H^+}}{dt} = \alpha \frac{dA}{dt}$$

$$= \frac{dA}{dt} \qquad \text{as } \alpha = 1 \text{ for NaOH.}$$

The titration, however, is complicated by the buffer capacity (BC) of the solution. This must be considered when nitrocatechol sulphate is used as substrate as it has a large buffer capacity (-8.3 at 3 mM) at pH 5.6. The pH-stat is run under Δt control, or with the proportional band set above zero, for this reason. Under these conditions specific aliquots of reagent are added at regular intervals but the frequency of addition and/or the amount added is proportional to $(pH-pH_p)$ or the difference between the actual pH and the pH set as the endpoint. As pH_p is approached the frequency and amount of base added decreases. The quantity of base released at each addition is equal to $-BC \left(\frac{dpH}{dt}\right)_o \Delta t_c$, where $\left(\frac{dpH}{dt}\right)_o = \frac{\Delta pH_c}{\Delta t_c}$ and is the theoretical rate at which the pH is changing and pH_c is the working pH. This means that the amount added is relatively independent of BC as $\left(\frac{dpH}{dt}\right)_o$ is approximately inversely proportional to BC. If the pH-stat were used with ΔpH_c control, i.e. the proportional band set at zero, the amount of base added in each addition would equal $-BC\Delta pH_c$. Thus, when BC is high,

large quantities are added and smooth titration curves are not produced.

Using Δt control, the drift in pH due to insufficient compensation of $\left(\frac{dpH}{dt}\right)_o$ by $\left(\frac{dM_{H+}}{dt}\right)_{average}$; the average rate of reagent addition, is given by

$$\left(\frac{dM_{H+}}{dt}\right)_{average} + BC\left(\frac{dpH}{dt}\right)_o = BC\,\frac{dpH}{dt} \qquad (17)$$

As

$$\left(\frac{dM_{H^+}}{dt}\right)_{average} = P(pH-pH_p)$$

where P is the proportional band setting; or, as above, the average rate of addition is proportional to the difference in pH, a differential equation can be obtained from (17) whose solution is

$$pH = pH_p + e^{Pt/BC}\int_o^t \left(\frac{dpH}{dt}\right)_o e^{-Pt/BC}dt \ .$$

When $\left(\frac{dpH}{dt}\right)_o$ is constant, that is, the reaction is zero order and the rate at which the pH changes is steady over the period of time measured,

$$pH = pH_p - \frac{BC}{P}\left(\frac{dpH}{dt}\right)_o\left(1 - e^{Pt/BC}\right)$$

From this equation is can be seen that when titration is initiated an exponential rise will be observed which is a function of the mechanics of the pH-stat not the reaction being measured. After this lag period, which will depend

on BC, the average slope of the recording will correspond to the reaction. With sulphatase A the reaction is not zero order and

$$\frac{dN_{H^+}}{dt} = v_O \, e^{-kt}$$

This does not affect the irregularities in the initial titration nor the dependence of the titration on BC. In this case, as was shown by Roy (1978)

$$M_{H^+} = \frac{-v_O}{k}\left(1 - e^{-kt}\right) + \frac{v_O}{k-\omega}\left(e^{-kt} - e^{-\omega t}\right)$$

where

$$\omega = \frac{x \cdot v_O}{BC \cdot \Delta pH_m} \qquad (\Delta pH_m = pH - pH_p \text{ and will be the proportional band setting})$$

The first term of this equation is the theoretical amount of reagent which is added to compensate for the sulphatase A reaction and the second term reflects the mechanics of the pH-stat. It is important to realize that for these reasons the early times of the pH-stat recording cannot be a true representation of the reaction being monitored and that the buffering capacity of nitrocatechol sulphate results in long lag times (i.e. approximately 1 minute at 5 mM) where the apparent rates measured will be less than the true rates. At lower nitrocatechol sulphate concentrations, of course, this effect is still present but the error it induces in the titration curve is much smaller and the lag time is shorter.

The equipment used in the present work was a slightly

modified assembly PHM26-ttl1-SBR2-ABU12 (Radiometer Ltd. Copenhagen). The reaction mixture was contained in a glass vessel jacketed at 37°C and with a side arm to allow additions without disturbing the recording of the reaction. The Radiometer G202C glass electrode and K401 calomel electrode were kept continually at 37°C in this vessel.

The burette had a capacity of 0.25 ml and was modified to give as nearly as possible an all-glass system. A glass burette tip was used with a small U-bend at the bottom to prevent leakage. A solution of approximately 0.007 M carbonate-free NaOH was used as titrant. This was standardized with twice recrystallised potassium biiodate in a carbon-dioxide free solution and in an atmosphere of nitrogen.

The recorder pen was driven by the burette through a rigid drive shaft rather than through the flexible drive used by Radiometer. To obtain reliable data at low substrate concentrations a gear box was fitted to this drive shaft which increased the rate of movement of the recorder pen by a factor of 6.24 but did not affect the actual rate of titration. Measurable slopes could therefore be obtained without depleting the substrate concentration. In 30 assays done in this way with the concentration of substrate varying between 0.1 and 0.5 mM the percentage of substrate remaining at 60 minutes was $86 \pm 8\%$.

The pH meter was always used on the expanded scale and was calibrated each day before use. The titrator was used without the delayed shut off and with the proportional band

set at 0.05. The burette was run at a speed which was approximately twice the rate of the reaction being measured. The reaction was recorded at a chart speed of either 7.5 cm min^{-1} (for initial velocities) or 1 cm min^{-1} (for activated velocities). Bovine serum albumin was not added to the reaction mixture as was suggested by Jerfy and Roy (1972) as it was found that the inactivation resulting from the leakage of Hg_2Cl_2 from the calomel electrode, which they describe, could be eliminated by changing the potassium chloride solution in the electrode daily.

Standard assay conditions refer to the pH-stat assay at 37°C using 10 ml 3 mM nitrocatechol sulphate, 0.1 M KCl adjusted to pH 5.6 in the pH-stat immediately before assay. The reaction is initiated by the addition of no more than 25 µl of enzyme solution. The buffer concentration of this solution is kept low to minimize the shift in pH which will occur when it is added to the reaction mixture. If this were not done an appreciable change in pH would be observed when the reaction was initiated and titration, and therefore recording of the actual reaction, would not begin until the pH had again reached 5.6. This is particularly important when assaying the modified enzyme as relatively large volumes may be needed to produce measurable velocity curves.

The assay method of Nicholls and Roy (1971) and Stinshoff (1972) in which the reaction is initiated by the addition of substrate rather than enzyme was investigated because it has the theoretical advantage that it avoids the changes in the polymerization state of the enzyme which occur by its dilution, as in the standard assay.

A small volume of concentrated substrate solution was added to the enzyme which had been diluted in 0.1 M KCl and equilibrated in the pH-stat vessel. Problems were encountered in keeping the nitrocatechol sulphate, in high concentrations, in solution and also with slight changes in the pH of the reaction mixture on addition. Generally more variance was found in replicate assays done this way and critical initial times were often lost. The method described above where the reaction was initiated by the addition of enzyme was therefore used throughout.

When initial velocities are being measured the reaction is followed for 3 minutes at a recorder speed of 7.5 cm min^{-1}.

When the activation of the substrate-modified enzyme is being determined the enzyme is added to the reaction mixture at zero time and titrated for a total of 60 minutes. The first 3 minutes are recorded at a speed of 7.5 cm min^{-1} and the remainder of the assay at 1 cm min^{-1}. If an activator is to be used it is added at 30 minutes and the reaction is allowed to proceed for the final 30 minutes.

2.12 Protonometric Assay

An assay method based on the same principles as the pH-stat assay but having much greater sensitivity was developed by Gutfreund and Hammond (1963). In this technique the course of the reaction is followed by measuring the change in potential of the glass electrode due to changes in the H^+ concentration in the reaction mixture. This method has been applied to the sulphatase A reaction by Roy (private communication) and has proven advantageous

over the pH-stat and spectrophotometric assays for measuring low reaction rates due to either small amounts of enzyme or enzyme having low activity. The method was used here to measure initial velocities of the modified enzyme preparation and consistent results were obtained with enzyme concentrations equivalent to 0.02 µg ml^{-1} native sulphatase A, an order of magnitude less than is required in the pH-stat.

A solution of 3 mM nitrocatechol sulphate, 0.1 M KCl is brought to pH 5.6 at 37°C and held at that temperature. For each assay 1 ml of this solution is added to the electrode vessel and allowed to equilibrate with stirring for 15 minutes. The stirrer was then turned off and a baseline recorded for 3 minutes. The enzyme was added and the solution stirred again for 10 seconds. After recording the reaction for 4 minutes 0.5 µl of HCl accurately standardized at approximately 0.02 M was added, again with stirring, to allow error due to minor differences in the buffer capacity of different assay mixtures to be reduced. The stirrer was turned off for the actual recording of the reaction as a smoother curve was produced in this way. This can be seen in the recordings shown in Figure 6, one of which was taken from an assay with continuous stirring (bottom progress curve) and the other as described above (top progress curve).

2.13 Spectrophotometric Assay

A. Qualitative Assay for Substrate-Modified Sulphatase A

This assay was used only for locating the enzyme

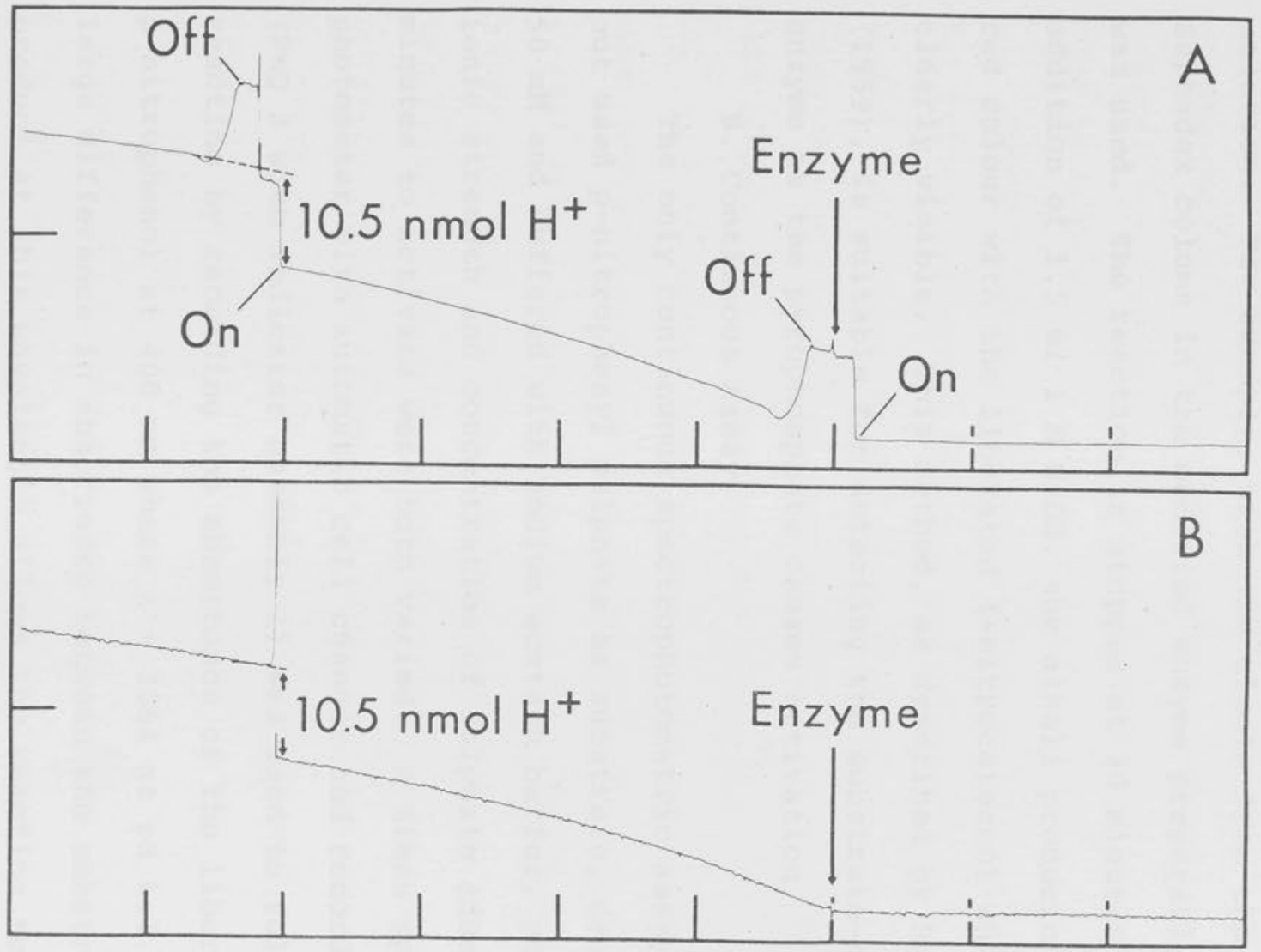


Figure 6 Recordings from protonometric assays of native sulphatase A demonstrating the effect of stirring on the recording. The assays are identical except that the lower curve (B) was obtained with continual stirring while the upper curve (A) was obtained, as described in the text, with stirring only when additions of enzyme or HCl were made.

activity in column eluates or filtrates. One ml of a solution consisting of 5 mM nitrocatechol sulphate, 0.25 mM $Na_4P_2O_7$, 0.25 M sodium acetate buffer, pH 5.0, 5% NaCl is incubated at 37°C with an aliquot of the enzyme solution. For example, with the eluate from the DEAE-Sephadex column in the modified enzyme preparation 10 µl was used. The reaction is stopped at 30 minutes with the addition of 1.5 ml 1 M NaOH, the alkali producing a dark red colour with the liberated 4-nitrocatechol which is clearly visible. This method, as described by Baum *et al* (1959), is suitable for detecting the substrate-modified enzyme as the pyrophosphate causes activation.

B. Continuous Assay

The only continuous spectrophotometric assays carried out used p-nitrophenyl sulphate as substrate, usually at 50 mM and buffered with sodium acetate buffer, pH 6.1. The ionic strength and concentration of sulphate added at 30 minutes to activate were both varied. A Ziess spectrophotometer with automatic cell changer and recorder (PMQ 3 with indicator assembly 1) was used to follow the reaction by recording the absorbance of the liberated p-nitrophenol at 400 nm where $\varepsilon = 3064$ at pH 6.1. The large difference in absorbance between the substrate and product at this wavelength allows the reaction to be followed continuously and this assay has the advantage over the pH-stat assay that, with a 6 cell cuvette holder and automatic changer, 5 assays can be run concurrently. Apart from saving time, this could possibly increase the precision in assays where the modified enzyme is being

activated. If a large volume of the standard reaction mixture, including the enzyme at the normal concentration, was incubated for 30 minutes and then aliquots of this solution added, along with the activator, to each of the five cuvettes the error due to small differences in the enzyme concentration in the different assays would be reduced. No greater precision, however, was found in assays done in this way.

Spectrophotometric assays might have been preferable for activation assays because the error in the recording of the early times of the pH-stat assay, due to the mechanics of the system (see section 2.11), would be eliminated. The curves observed after the addition of SO_4^{2-} to activation assays using p-nitrophenyl sulphate as substrate were very similar to those observed with the pH-stat assays and although only a limited number of these assays was done the data did not appear to be more precise when fitted to kinetic equations. This method was not routinely applied therefore because nitrocatechol sulphate, the preferred substrate, could not be used and no other advantage was evident.

2.2 CALCULATIONS

These calculations are described for the pH-stat assay as it was used most often and as this clarifies the explanation. Calculations using other recordings, i.e. from the spectrophotometric or protonometric assays, are done in a similar manner.

2.21 Initial Velocity

Readings were taken from the recording between 1 and 3 minutes at 8 second intervals and the initial velocity (v_o) calculated from these points. The method used was described by Roy (1978) and follows the rate equation

$$(s_o - s) = \frac{v_o}{k^*} \quad (1 - e^{-k^* t}) + C \qquad (2.1)$$

where $k^* = k \cdot \frac{s_o}{K+s_o}$ and k is the rate constant for the formation of substrate-modified enzyme. The empirical constant, C, is necessary when the pH-stat is used as the recording cannot be extrapolated through the origin for the reasons given in section 2.11. This exponential was fitted by the method of Guggenheim (1926). Readings were taken from the assay recording at a series of times t_1, t_2, ... and $t_{1+\tau}$, $t_{2+\tau}$, ... where τ is constant. These sets of readings; u_1, u_2, ... and $u_{1+\tau}$, $u_{2+\tau}$, ..., are proportional to the concentration of product present and therefore to $(s_o - s)$ or the decrease in substrate concentration. If each set of readings is fitted to equation (2.1) and the difference between the two taken then equation (2.2) is produced.

$$(u_{t+\tau} - u_t) = \frac{v_o}{k^*} e^{-k^* t} \quad (1 - e^{-k^* \tau}) \qquad (2.2)$$

Converting this to the logarithmic form gives

$$\ln (u_{t+\tau} - u_t) = -k^* t + \ln \frac{v_o}{k^*} (1 - e^{-k^* \tau})$$

A plot of $\ln (u_{t+\tau} - u_t)$ versus t will therefore have a slope

equal to k* and the intercept will give v_o.

The standard deviation found in v_o in replicate pH-stat assays calculated in this way is less than 5% (e.g. 206 $\pm$ 5 µmoles min^{-1} mg^{-1} in 8 assays) and less than 5% of the substrate was hydrolyzed in an assay.

2.22 Activated Velocity

The rate observed between 20 and 30 minutes after the addition of the activator, or when the rate had become linear, was taken as the activated velocity. Readings were taken at 1 minute intervals over this time period and a line fitted through them by linear regression. The standard deviation found in this velocity over six replicate assays was 7% (e.g. 3.72 $\pm$ 0.26 µmoles min^{-1} mg^{-1}).

The error in this measurement was not increased when the gear box was used to increase the apparent velocity (e.g. 8.43 $\pm$ 0.24% min^{-1} in six replicate assays).

2.23 Extent of Modification

The fraction of substrate-modified enzyme present in a sulphatase A solution was usually calculated by comparing the initial velocity (v_o) of the solution after inactivation to that of the native enzyme used in its preparation. The initial activity observed was assumed to result from native sulphatase A remaining in the preparation as its response to the addition of effectors was identical to that of the native enzyme and also because it could be further reduced by incubating the modified enzyme with substrate a second time. It was therefore used as a measure of the concentration of enzyme which had not been modified. This method was preferred to the one described

below because initial velocities could be determined more precisely. When insufficient enzyme was present to get a reliable value for v_0 an alternative method was used. The amount of residual native enzyme was calculated from the ratio of the initial to the activated slope as described by Nicholls and Roy (1971). Both the native and substrate-modified enzyme are assayed in 3 mM nitrocatechol sulphate for 60 minutes with the addition of 3 mM K_2SO_4 at 30 minutes. By taking the ratio of the slope between the first 2 to 3 minutes of the reaction, and the slope between 20 and 30 minutes after the addition of sulphate for native sulphatase A and comparing this ratio to that obtained with the modified sulphatase A preparation the amount of native enzyme remaining can be determined. This calculation assumes that the initial activity is due only to native enzyme whereas the activity found after sulphate has been added is due to both the native and modified enzyme present in the original solution. The ratio of 2 to 3 minute rates to 20 to 30 minute sulphate activated rates for native enzyme was 2.6 to 2.9 depending on the particular batch of enzyme used. For comparison, Nicholls and Roy found a ratio of 3.5 for ox liver sulphatase A assayed with 1 mM nitrocatechol sulphate, pH 5.6 and activated with 1 mM K_2SO_4.

Both of the above methods gave similar values for the amount of residual native enzyme in preparations of the modified enzyme. For example, a solution which contained 5% native enzyme as determined by comparing initial velocities $\left(\frac{v_0(\text{modified})}{v_0(\text{native})} = \frac{10}{203} = 0.05\right)$ gave a value

of 6% native enzyme by the ratio method $\left(\frac{v(\text{initial})}{v(SO_4^{2-}\ \text{activated})} \times \frac{1}{2.8} = \frac{2}{12.68} \times 0.36 = 0.06\right)$.

2.3 DETERMINATION OF PROTEIN CONCENTRATION

2.31 Differential Refractometry

The refractive index difference of the native and substrate-modified enzymes and their solvents were measured with a Brice-Phoenix Model BP-2000 Differential Refractometer which had been calibrated at 546 nm with dried KCl to find the refractometer constant. The enzyme solutions were dialysed against 5 mM Tris-HCl, pH 7.4, I=0.1 and this buffer was used as a blank. Five readings were taken at each position and averaged. The difference between these readings, corrected for the buffer, gave the total slit image displacement of the solution in instrument units. This, multiplied by the refractometer constant, gives the refractive index difference. Assuming a refractive index of 1.84×10^{-3} dl g^{-1} (Perlmann and Longsworth, 1948) the concentration of protein can be calculated.

2.32 Absorbance at 280 nm

The concentration of enzyme was routinely determined spectrophotometrically in a Zeiss PMQ 3 spectrophotometer. The specific absorbance of the substrate-modified enzyme was calculated by first determining the concentration of protein by differential refractometry, as given above, and then measuring the absorbance at 280 nm. The $E^{1\%}_{280\ nm}$ was calculated to be 7.0 for both the modified and native

form of the enzyme at pH 7.4. This value is in agreement with that obtained by Nichol and Roy (1965) for native sulphatase A in the same manner.

2.33 Fluorometric Determination

When the amount of enzyme to be measured was insufficient to give a reliable absorbance reading a fluorometric technique described by Benson and Hare (1975) was used to determine the protein concentration. The method involves reacting the protein solution with o-phthalaldehyde (Fluorópa) in the presence of 2-mercaptoethanol. This compound will react with primary amines to give products which will fluoresce with λ_{ex} = 340 nm, λ_{em} = 455 nm. The reagent, which must be made up daily, was prepared by dissolving 20 mg o-phthalaldehyde in 25 µl 95% ethanol and making the volume up to 25 ml with 0.4 M boric acid which had been brought to pH 9.7 with KOH and contained 0.2% 2-mercaptoethanol. An equal volume of this reagent and the protein solution were mixed (0.5 ml each) and the fluorescence of the resulting solution measured 1 minute after mixing. An Aminco-Bowman Spectrofluorimeter was used with two $^{3}/_{16}$ inch slits and the photomultiplier set to 1 mm which gave reproducible readings for the range of protein concentrations being measured, i.e. 10-40 µg ml^{-1}. In order to determine a constant to convert the measured fluorescence to the concentration of enzyme, standards were run of both native and modified enzyme whose concentrations had been determined spectrophotometrically before dilution and of a bovine serum albumin solution of known concentration.

The latter was measured to obtain a ratio of the fluorescence produced by the enzyme to that of the BSA so that in subsequent determinations a sample of BSA only need be run as standard and not the less abundant enzyme. It is necessary to run a standard with each set of measurements as the reagent is prepared fresh each day and differences in concentrations may occur. As the solution gradually deteriorates differences would also be observed over the course of a day using the same solution. The ratio of fluorescence of enzyme to fluorescence of BSA was 3.2 ± 0.2 as determined by averaging 3 readings from 3 samples of enzyme and 3 samples of BSA. No difference was found in the amount of fluorescence produced by native and modified enzyme.

2.4 SUBSTRATES

2.41 Nitrocatechol Sulphate

Dipotassium 2-hydroxy-5-nitrophenyl sulphate, commonly called nitrocatechol sulphate, was synthesized by the method of Roy (1958) which involves the oxidation of p-nitrophenol with peroxydisulphate. A suspension of 150 g p-nitrophenol in 5 l H_2O containing 350 g KOH and 350 g $K_2S_2O_8$ was kept at 37°C for 4 days and then worked up as directed. It was purified by converting the dipotassium salt to the monopotassium salt as given by Dodgson and Spencer (1956a) and recrystallizing from 75% ethanol until a test for SO_4^{2-} was negative and no nitrocatechol was detected. The latter was measured spectrophotometrically using a solution in 0.1 M NaOH and a Varian SuperScan 3. The region between 350 and 600 nm

was scanned. This spectra was then compared to that of a sample of nitrocatechol sulphate of known purity. The key wavelengths were 405 nm, the λ_{max} for nitrocatechol sulphate where ε = 18 900 in 0.1 M NaOH and 510 nm, the λ_{max} for nitrocatechol where ε = 12 600 in 0.1 M NaOH. A 14% yield was obtained using this method.

Nitrocatechol was obtained by acid hydrolysis of nitrocatechol sulphate. Reaction conditions identical to those given above were used to prepare the nitrocatechol sulphate but it was not separated from the reaction mixture nor purified. After the first ether extraction, to remove the unreacted phenol, the pH was dropped to 1 and the solution kept at 37°C for several hours. The nitrocatechol was then extracted with 6 volumes of 250 ml diethyl ether. After evaporating to dryness, the product was recrystallized from water until its absorbance spectrum was identical to that of a sample of nitrocatechol of known purity. In 0.1 N NaOH ε_{NC} equals 12 600 at 510 nm.

2.42 Other Aryl Sulphates

The p-nitrophenyl sulphate used was synthesized by Dr. A.B. Roy by the method of Burkhardt and Wood (1929) and recrystallized before use from 80% ethanol containing 0.01 M $KHCO_3$.

The other substrates used had also been synthesized by Dr. A.B. Roy. L-ascorbate 2-sulphate by the method of Quadri *et al* (1973), 4-methylumbelliferone sulphate by the method of Sherman and Stanfield (1976) and nitroquinol sulphate by a modification of the method of Smith (1951). This latter method involves a persulphate oxidation of

m-nitrophenol and is similar to the method used to produce nitrocatechol sulphate.

2.43 Nitrocatechol [^{35}S]Sulphate

The labelled substrate was synthesized from 4-nitrocatechol and chlorosulphonic acid by the method of Flynn *et al* (1967). To 1.5 g 4-nitrocatechol in 3.2 ml CS_2 and 3.7 ml N,N-dimethylaniline were added 5 mCi chloro[^{35}S]sulphonic acid mixed with 0.7 ml of redistilled chlorosulphonic acid. For different preparations three lots of labelled chlorosulphonic acid (Radiochemical Centre, Amersham) were used having specific activities of 22, 61, and 31 mCi mmole^{-1} when originally synthesized. The reaction mixture was worked up in the usual way and a 50% yield of nitrocatechol sulphate was obtained with a specific activity of approximately 0.1 Ci mole^{-1}. Again the purity of the product was determined spectrophotometrically.

2.5 DETERMINATION OF RADIOACTIVITY

Radioactivity was counted on a Packard Model 3255 Tri-Carb Liquid Scintillation Spectrometer System. The preset $^{14}C/^{14}CQ$ channel was used as it gave more reproducible counts than setting the adjustable discriminators by the method given in the manual using a sample of ^{35}S. Counting efficiency was determined by the external standard channels ratio method. The system was calibrated with standards of $^{35}SO_4$ in aqueous solution, their activities having been calculated from the activity given by Radiochemical and the length of time which had elapsed before counting. These standards were counted under various quenching conditions and a plot made of the sample

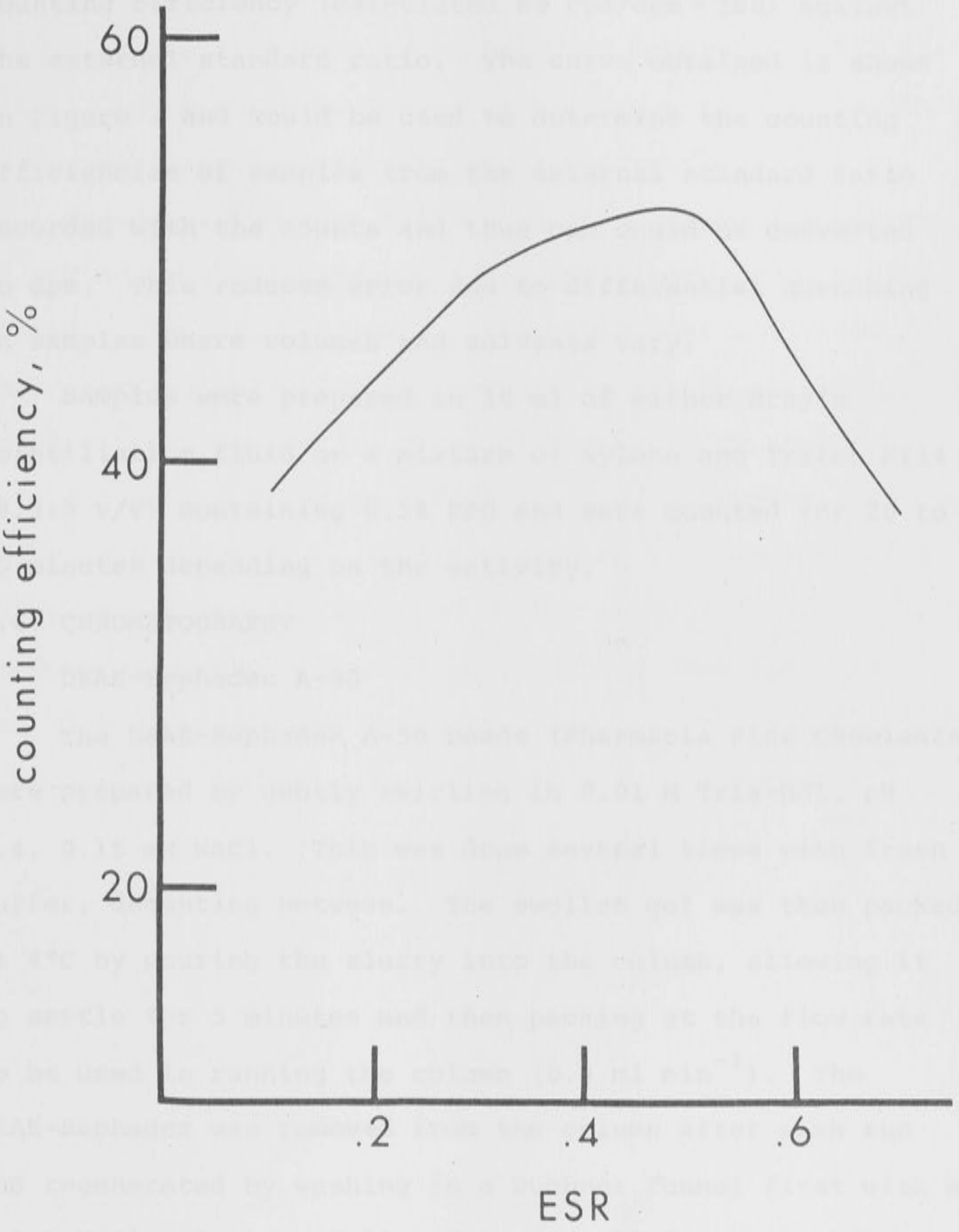


Figure 7 Standard curve for converting cpm to dpm for ^{35}S. The counting efficiency, calculated as the ratio of observed counts per min to known disintegrations per min, is plotted as a function of the external standard ratio given by the scintillation counter.

counting efficiency (calculated as cpm/dpm × 100) against the external standard ratio. The curve obtained is shown in Figure 7 and could be used to determine the counting efficiencies of samples from the external standard ratio recorded with the counts and thus cpm could be converted to dpm. This reduces error due to differential quenching in samples where volumes and solvents vary.

Samples were prepared in 10 ml of either Bray's scintillation fluid or a mixture of xylene and Triton X114 (8.3:5 v/v) containing 0.5% PPO and were counted for 20 to 60 minutes depending on the activity.

2.6 CHROMATOGRAPHY

DEAE-Sephadex A-50

The DEAE-Sephadex A-50 beads (Pharmacia Fine Chemicals) were prepared by gently swirling in 0.01 M Tris-HCl, pH 7.4, 0.15 mM NaCl. This was done several times with fresh buffer, decanting between. The swollen gel was then packed at 4°C by pouring the slurry into the column, allowing it to settle for 5 minutes and then packing at the flow rate to be used in running the column (0.4 ml min^{-1}). The DEAE-Sephadex was removed from the column after each run and regenerated by washing in a Buchner funnel first with a 0.1 M NaCl solution, followed by distilled water until neutral pH was regained, and then with the above buffer.

Sephadex

Sephadex G-25 was prepared by standing in several changes of 0.01 M Tris-HCl, pH 7.4 for approximately 4 hours. It was then chilled to 4°C and packed in the column required at this temperature. It was further equilibrated

by running twice the bed volume of buffer through the column at the flow rate to be used for separation (1-2 ml min^{-1}). Once used the column was washed with 0.01 M NaOH and re-equilibrated with the above buffer.

These columns were calibrated by applying a solution of bovine serum albumin and K_2SO_4 and eluting with 0.01 M Tris-HCl, pH 7.4, 0.15 NaCl. Fractions were collected, their absorbance at 280 nm measured, and $BaCl_2$ and HCl added to test for SO_4^{2-}. Figure 8 shows the separation obtained on a 1.5 × 26 cm column of 20 ml of BSA (0.2 mg ml^{-1}) in 50 mM K_2SO_4. As nitrocatechol and nitrocatechol sulphate are loosely adsorbed to the Sephadex their movement down the column is retarded and they elute far behind the protein and sulphate.

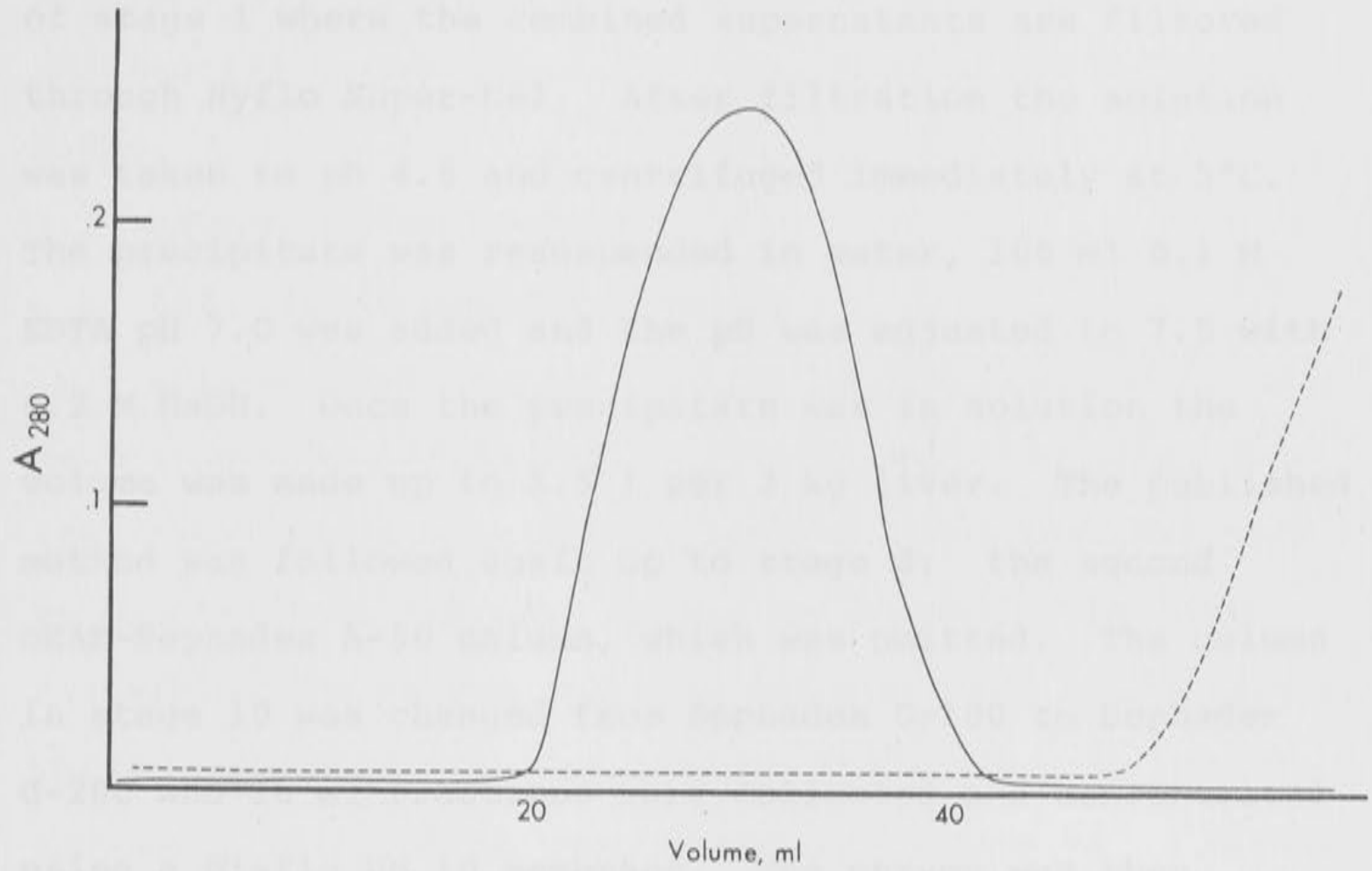


Figure 8 The elution of 20 ml of 0.2 mg ml^{-1} BSA in 50 mM K_2SO_4 from a 1.5 × 26 cm column of Sephadex G-25 at 4°C. The absorbance of the fractions at 280 nm was measured to locate the BSA (——) and then $BaCl_2$ and HCl were added to determine the SO_4^{2-} content (----).

3. NATIVE SULPHATASE A

3.1 PREPARATION FROM OX LIVER

Sulphatase A was extracted and purified from fresh ox liver by a modification of the method of Nichol and Roy (1964). The method was followed as given to the end of stage 1 where the combined supernatants are filtered through Hyflo Super-Cel. After filtration the solution was taken to pH 4.5 and centrifuged immediately at 5°C. The precipitate was resuspended in water, 100 ml 0.1 M EDTA pH 7.0 was added and the pH was adjusted to 7.5 with 0.2 M NaOH. Once the precipitate was in solution the volume was made up to 1.5 l per 3 kg liver. The published method was followed again up to stage 8; the second DEAE-Sephadex A-50 column, which was omitted. The column in stage 10 was changed from Sephadex G-100 to Sephadex G-200 and 10 ml fractions were collected and concentrated using a Diaflo UM 10 membrane. The enzyme was then dialysed against 0.01 M Tris-HCl pH 7.4, I=0.1 overnight and stored at 5°C. Its concentration was determined spectrophotometrically (see section 2.32) and its specific activity was measured by the pH-stat assay (see section 2.11). An average yield of 0.5 mg kg^{-1} was obtained with a specific activity range of 203-248 µmoles min^{-1} mg^{-1} in six independent preparations. These activities are considerably greater than previously reported values, eg. 140 µmoles min^{-1} mg^{-1} (Nichol and Roy, 1964). This results from the calculation of initial velocities and controlling the ionic strength of the reaction mixture by the addition of 0.1 M KCl (see section 2.21).

The modifications in the preparation do not change the basis of separation but improve on the conditions used. For instance, the enzyme solution was centrifuged immediately after the pH was dropped to 4.5 and then the pH adjusted to 7.5 as quickly as possible because the enzyme is acid labile. No advantage was found in using a second DEAE-Sephadex A-50 column so this step was omitted and better separation was obtained on Sephadex G-200.

3.2 DETERMINATION OF PURITY

3.21 Sedimentation Velocity

Native enzyme in Tris buffer pH 7.4 (2 mg ml^{-1}) was centrifuged at 52 000 rpm at 19°C in a Spinco Model E analytical ultracentrifuge. An An-D rotor was used with an aluminium-filled epon double sector cell, one side containing the enzyme solution and the other buffer alone. These conditions are identical to those given by Nichol and Roy (1964). Five photographs were taken at 16 minute intervals starting immediately after the set speed had been reached. Each photograph showed a single symmetrical peak and so no evidence of heterogeneity in the preparation.

The value of s was calculated from the rate of movement of the maximum ordinate as given by

$$s = \frac{d \ln x}{dt} \times \frac{1}{\omega^2}$$

where x is the distance of the maximum ordinate from the centre of the rotor. This value is then corrected to 20°C in water using the empirical relationship given by

Svedberg and Pedersen (1940).

$$s_{20,w} = s_t \times \frac{\eta^o_t}{\eta^o_{20,w}} \frac{(1 - \bar{v}_{20}\ \rho^o_{20})w}{(1 - \bar{v}_t\ \rho^o_t)}$$

A $s_{20,w}$ of 6.1 S was obtained which is comparable to the value of 6.3 S found by Nichol and Roy for the native enzyme at 2 mg ml^{-1} in diethylbarbiturate buffer pH 7.5, I=0.1 and corresponds to a molecular weight of 110 000.

3.22 Zone Electrophoresis

Electrophoresis of the sulphatase A on cellulose acetate strips also gave no evidence of heterogeneity. A Beckman Microzone electrophoresis apparatus was used with Beckman electrophoresis membrane no. 324330 as support and Veronal buffer pH 8.6, I=0.055 as the conducting medium. Five µl of the enzyme solution (0.4 mg ml^{-1}) was applied to the support and it was run at a constant voltage of 250 V. After one hour the strip was removed, fixed in 20% sulfosalicyclic acid for 2 minutes, stained with 0.25% Coomassie Blue in water for 5 minutes and then destained with several changes of distilled water. A single band was observed indicating that there was no contaminating protein present which had a mobility different from that of sulphatase A under these conditions.

3.3 SUBUNIT STRUCTURE

3.31 Sulphatase A from Other Sources

It has become evident in the studies of arylsulphatases from many mammalian sources that the smallest unit of enzyme present under non-denaturing conditions will break down into subunits if denaturants are

added to the solution. The molecular weights of these subunits have generally been determined by sodium dodecyl sulphate polyacrylamide gel electrophoresis and a brief summary of this data is given in Table 6. As is seen in the table most of the enzymes studied are composed of subunits of identical size although a few have been studied which break down into subunits of unequal size. Draper *et al* (1976) note that with the human liver enzyme the non-identical subunits may result from modification during its preparation. This would not be unexpected considering the abundance of hydrolytic enzymes in liver. James and Austin (1979) also obtained subunits of unequal size from their preparation of human liver sulphatase A. They further investigated the subunits by peptide mapping and found that the two maps were very similar, the only difference being that the larger subunit produced two more spots. This suggests the possibility that the two subunits may be identical *in vivo* and that the smaller one is derived from the larger during isolation. It does not necessarily indicate that this is not the natural state of the enzyme.

More generally, it has been proposed by Moser *et al* (1972), on the basis of the general lack of sulphatases in multiple sulphatase deficiency, that all of these enzymes may contain a common subunit. Draper *et al* (1976) further suggest that the catalytic subunit in all sulphatases may be similar and that the second regulatory subunit differs and may be responsible for conferring the different specificities to the different enzymes. It

TABLE 6

SUBUNIT STRUCTURE OF SULPHATASES A

Source	Molecular Weight, pH 7.5	Molecular Weight of Subunits	
human liver	134 000[f]	69 000 57 000	(Collins *et al*, 1967; James and Austin, 1979)
	104 500[b]	66 000[d] 53 000	(Draper *et al*, 1976)
		59 000[a] 49 000	(Draper *et al*, 1976)
human urine	100 000[f]	50 000[f]	(Stevens *et al*, 1975)
rabbit liver	147 000[f]	70 000[a]	(Lee and Van Etten, 1975a)
sheep brain	122 000[f]	63 000[a]	(Balasubramanian and Bachhawat,1975)
rabbit testis	110 000[f]	55 000[a]	(Yang and Scrivastava, 1976)
ox liver	107 000[c]	24 000[e] 50 000[e]	(Nichol and Roy, 1964, 1965) (Roy and Jerfy, 1970)

a SDS polyacrylamide gels
b sedimentation equilibrium
c sedimentation velocity
d sedimentation velocity of guanidine hydrochloride treated enzyme
e sedimentation velocity of SDS treated enzyme
f gel filtration

should be noted that no immunological evidence exists to support this.

With the human liver enzyme a small amount of material having a molecular weight of approximately half that of the other subunits was also observed.

3.32 Ox Liver Sulphatase A

A. Methods

The method used was that given by Shapiro, Viñuela and Maizel (1967) and tested by Weber and Osborn (1969). Gels were polymerized in 9 cm long tubes having an inner diameter of 0.5 cm and run in an Ames disc electrophoresis apparatus Model 1200 with an Ames electrophoresis constant rate source Model 100 A. Gels were made up immediately before use as described to give final concentrations of 10% acrylamide, 0.03% bisacrylamide, 0.1% SDS, 0.1% 2-mercaptoethanol, and either 0.1 M sodium phosphate buffer pH 7.0 or 0.1 M sodium acetate buffer pH 5.0. The acrylamide was polymerized by the addition of ammonium persulfate and N,N,N',N'-tetramethylethylenediamine.

Protein samples of approximately 0.3 mg ml^{-1} were incubated in 0.01 M sodium phosphate buffer, pH 7.0, or 0.01 M sodium acetate buffer, pH 5.0, 1% SDS and 1% 2-mercaptoethanol for 3 hours at 37°C. They were then dialysed at room temperature overnight against 0.01 M sodium phosphate buffer, pH 7.0, or 0.01 sodium acetate buffer, pH 5.0, 0.1% SDS and 0.1% 2-mercaptoethanol. Fifty µl of the protein solution was then mixed with 5 µl 0.05% bromophenol blue, 1 drop of glycerol, 5 µl 2-mercaptoethanol and 50 µl of the dialysis buffer and

applied to a gel. The buffer used in both compartments of the electrophoresis apparatus was identical to that in the gels. The gels were run at a constant current of 8 ma for 3 to 15 hours.

A standard curve showing the relationship between molecular weight and mobility was made using bovine serum albumin, ovalbumin, glyceraldehyde phosphate dehydrogenase, pepsin, subtilisin and lysozyme and the molecular weights given for these proteins or their subunits in Weber and Osborn.

A sample of native sulphatase A was also digested with neuraminidase (mucopolysaccharide N-acetyl-neuraminidase EC 3.2.1.18, Behringwerke AG) in 0.05 M sodium acetate buffer, pH 5.5, 0.9% NaCl, 0.1% $CaCl_2$, as recommended by Behringwerke. Approximately 150 neuraminidase units were added per mg sulphatase A and the solution was incubated for 3 hours at 37°C. An aliquot was then tested by electrophoresis on cellulose acetate, as given above, with native enzyme as marker to ensure that the digestion had been complete and all of the sialic acid had been removed. The remaining solution was then dialyzed against the appropriate SDS, 2-mercaptoethanol solutions and incubated as before.

Only the molecular weights of the protein units produced could be determined as the subunits aggregate on removal of the SDS thus precluding measurement of their activity or binding. Since SDS is a powerful inhibitor of sulphatase A the activity of the subunits could not be measured in its presence.

The relative concentration of protein present in the observed bands was measured as the decrease in transmitted light across the gels once they had been stained. A Schoeffel spectrodensitometer Model SD3000 was used for this purpose with a double beam of light set at 540 nm. A gel containing no protein but having been stained in the same manner as the other gels was used as a blank in order to lower the baseline caused by the high degree of background staining. The gels were scanned automatically and the absorbance plotted by a Schoeffel density computer Model SD300. The ratio of the areas under the peaks was taken as a measure of the relative protein concentration.

B. Results

The results of the experiments are summarized in Table 7 and Figure 9. When the gels were run at pH 7.0 for 5 hours a single band was observed, the mobility of which corresponded to a protein of molecular weight 48 000. Under these conditions, therefore, the monomeric form of the enzyme, which has a molecular weight of 110 000, has been split into two subunits of identical size. If the gels were run for 15 hours, two bands were observed having molecular weights of 51 000 and 46 000. The amount of protein in the upper band (molecular weight 51 000) was 2.3 times greater than that in the lower band (molecular weight 46 000). The enzyme which had been digested with neuraminidase was run under the same conditions for 15 hours. After destaining only one band was present corresponding to a molecular weight of 39 000 which is slightly less than that of the lower band found without the neuraminidase digestion.

TABLE 7

OX LIVER SULPHATASE A

Native sulphatase A was run on SDS polyacrylamide gels under the conditions described in the text and given below. A minimum of four gels were run under each set of conditions. The numbers given in brackets beside the protein ratios are the number of gels measured.

conditions	mobility	molecular weight	ratio of protein in top to bottom band
pH 7.0			
5 h	0.232±0.009	48 000	
15 h	0.195±0.007 0.283±0.008	51 000 46 000	2.3±0.2 (3)
neuraminidase treated 15 h	0.340±0.013	39 000	
pH 5.0			
3 h	0.239±0.006 0.267±0.007	50 250 47 000	2.8±0.2 (3)
neuraminidase treated 3 h	0.249±0.012 0.296±0.013	49 000 43 500	2.5 (1)
10 h	0.213±0.010 0.236±0.010	54 000 51 000	2.5±0 (2)
neuraminidase treated 10 h	0.215±0.012 0.248±0.013	53 500 49 750	3.0 (1)

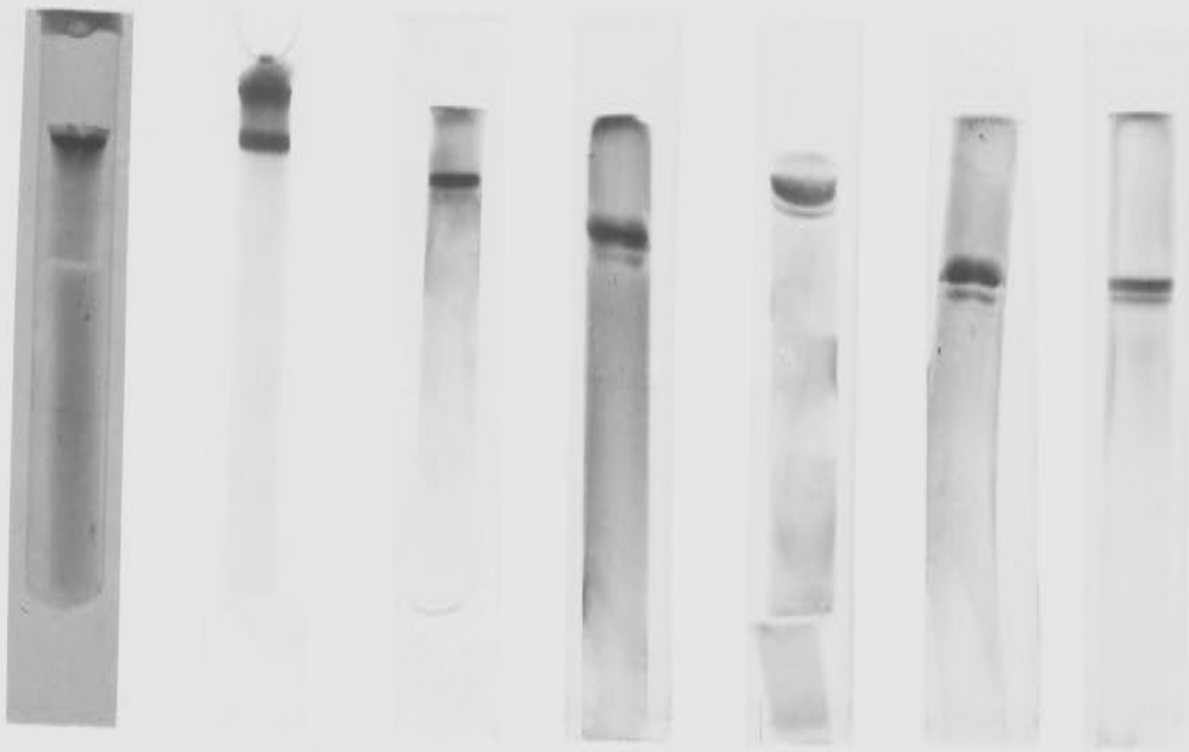


Figure 9 SDS polyacrylamide gels of sulphatase A after:

A 5 h electrophoresis at pH 7.0

B 15 h electrophoresis at pH 7.0

C 15 h electrophoresis at pH 7.0 after neuraminidase treatment

D 3 h electrophoresis at pH 5.0

E 3 h electrophoresis at pH 5.0 after neuraminidase treatment

F 10 h electrophoresis at pH 5.0

G 10 h electrophoresis at pH 5.0 after neuraminidase treatment

At pH 5.0 two bands were observed after electrophoresis for 3 hours. The molecular weights of these bands were 50 000 and 47 000 which correspond to those found at pH 7.0. The ratio of protein in the two bands was also comparable to that found at pH 7.0, the upper band containing 2.8 times the amount of protein in the lower. A longer electrophoresis time did not lead to further division of the two bands nor to a change in the ratio of the two. Treatment with neuraminidase did not alter this pattern when the gels were run for either 3 or 10 hours.

3.33 Discussion

There is some uncertainty in the literature as to the subunit composition of the ox liver sulphatase A and the conditions under which it dissociates. Nichol and Roy (1966) treated the enzyme at room temperature for two hours in 0.02 to 0.05% SDS solutions at pH 7.5 and then examined the solutions by analytical ultracentrifugation. Apart from micelles of SDS having a s value of approximately 1, only one peak was observed which had a s value of 6. This material corresponds to the monomer of sulphatase A. As the pH was lowered to 5.0, material of s 3.6 appeared and at the higher SDS concentration was the dominant form of enzyme present. The apparent molecular weight of the s 3.6 material, obtained using values of s and D* from sedimentation velocity experiments,is 60 000. This value, however, is for the SDS-protein complex. In order to calculate the molecular weight of the corresponding protein subunit the amount of SDS bound in the complex must be known. This value was estimated from a plot of the fraction

of intact tetramer versus the SDS to protein ratio by extrapolation to zero tetramer concentration, and a molecular weight of 24 000 was assigned to the protein subunit. As Nichol and Roy point out, there is some question about this value as there is evidence to suggest that all of the assumptions used in the calculation are not valid.

Roy and Jerfy (1970) also studied the dissociation of the ox liver sulphatase A into subunits under various conditions. At SDS concentrations greater than 0.02% they observed, using equilibrium centrifugation, a single molecular weight species which contained, using the SDS content of Nichol and Roy (above), a protein of molecular weight 50 000. They also studied the effect of 8 M urea and found that at pH 5.0 two incompletely resolved boundaries having s_{20} values of 1.1 S and 2.0 S were observed suggesting a heterogeneous population of small segments of the enzyme. At pH 8.0 neither urea nor dithiothreitol had any effect on their own but produced dissociation when present together. A subunit of molecular weight 55 000 was the predominate species observed although a small amount of 27 000 molecular weight material was also detected. It was suggested on the basis of this data that the monomeric enzyme may be composed of four subunits of molecular weight 27 000; two joined by disulphide bridges to give a 50 000 molecular weight unit and then two of these units joined by hydrogen and/or hydrophobic interactions to give the monomer.

Jerfy and Roy (1974) also observed that the ox liver sulphatase A broke down into subunits having a molecular weight of 59 000, as determined by sedimentation equilibrium, if azo groups were introduced into the enzyme by treatment with reagents such as 4-sulphodiazobenzene. These subunits showed no catalytic activity and their formation was a function of the ratio of enzyme to reagent and so to the degree of azotisation. It was suggested that the split resulted from interference to the hydrophobic interactions between the two subunits.

The data from the ultracentrifuge study (Nichol and Roy) indicated that dissociation did not occur at pH 7.0 from the action of SDS alone. It is possible, therefore, that with the SDS polyacrylamide gels the addition of the reducing agent is essential to the dissociation. However, as the incubation for the electrophoresis was much more severe (1% SDS/37°C/3 hours compared to 0.05% SDS/20°C/ 2 hours) it cannot be assumed that SDS would have no effect on its own. The fact that the molecular weights of the bands observed at pH 5.0, a pH where SDS alone was found to cause the break down of the enzyme, were comparable to those observed at pH 7.0 suggests that there is no difference in the mechanism of the dissociation at the two pH's.

The results obtained after electrophoresis for 5 hours at pH 7.0 indicate that sulphatase A is composed of two subunits of molecular weight 48 000. This agrees with the observations made by Roy and Jerfy when they treated the enzyme with SDS except that there was no evidence for the

existence of 27 000 molecular weight units on the polyacrylamide gels. The observation of these small subunits might have been expected from the action of the 2-mercaptoethanol on disulphide bonds if the hypothesis given above is correct. It should be noted that only small amounts were observed in the sedimentation equilibrium studies and they may not have been present in sufficient concentration to be detected on the gels, although this seems unlikely.

When the gels were run for 15 hours at pH 7.0 or for 3 or 10 hours at pH 5.0 two bands were observed corresponding to material of molecular weight 50 000 and 47 000. As the molecular weights and distribution of protein between the two bands were comparable at both pH's, the subunits produced in each case are probably identical.

The evidence suggests that the separation of the two dissimilar subunits relies more on a difference in their charge than on a difference in their molecular weight. A situation similar to that found at pH 7.0 has been noted by Herrmann *et al* (1973) with yeast phosphofructokinase. With this enzyme a single band was observed after electrophoresis for 5 hours using the technique given above, but if the run was prolonged to 15 hours two bands became apparent. These two subunits were shown to be non-identical by their reaction with antiserum. The fact that the normal electrophoresis time is insufficient to separate the subunits of the phosphofructokinase and those of sulphatase A at pH 7.0 suggests that they have a very similar molecular weight but differ in their charge. This would explain why

only a single molecular weight species was detected by ultracentrifugation with the ox liver sulphatase A treated with SDS. It also would explain why the two subunits were separated in 3 hours at pH 5.0 but not at pH 7.0 if the charge difference were greater at the lower pH.

It had previously been shown that treatment of the intact sulphatase A with neuraminidase completely removed the sialic acid and reduced its electrophoretic mobility (Graham and Roy, 1973; Goldstone *et al*, 1971). Also, Stevens *et al* (1976) had observed different isoenzymes of human liver sulphatase A, some of which apparently differed only in their sialic acid content. Isoelectric focusing of the purified enzyme gave six bands having pI's in the range 4.4 to 4.9. Neuraminidase treatment reduced the number of bands by converting bands 4-6 to bands 1-3. They suggest that the presence of these isoenzymes is due to a series of post-synthetic modifications of the enzyme which include sialylation.

It seemed possible that the small difference in mobility observed in the present work could result entirely or partially from differences in the sialic acid content of the subunits. When enzyme which had been digested with neuraminidase was run under identical conditions at pH 7.0 for 15 hours only one band was observed. This suggests either that two enzymes are present which differ in their sialic acid content or that the difference occurs between the two subunits in one enzyme molecule.

The situation at the lower pH was quite different. At pH 5.0 only 3 hours of electrophoresis were required to separate the two subunits and neither electrophoresis for a longer time nor treatment with neuraminidase altered the banding pattern. The molecular weights of the two subunits are comparable to those observed at pH 7.0, however. This suggests that the difference between the subunits is accentuated at the lower pH, which implies a difference in charge. Further, as neuraminidase had no effect at this pH it appears that the separation reflects more than a difference in sialic acid content of the subunits. The effect of neuraminidase at the higher pH may have been fortuitous, therefore, and does not mean the difference between the two subunits has been eliminated. While it is likely that the sialic acid content does vary, it seems that at pH 7.0 the two desialylated units have very similar charges while at pH 5.0 they do not.

The possibility also exists that one subunit was derived from the other either *in vivo* or sometime during or after the initial extraction of the enzyme from liver. This would be very likely if the only difference between the two was in their sialic acid content. Graham and Roy (1973) have demonstrated that removal of the sialic acid residues from ox liver sulphatase A does not affect the enzyme's activity and therefore their loss in some molecules would not be detected. Other slight alterations may also have occurred which do not affect the catalytic activity and are not sufficiently large to be detected in most physical methods. A situation similar to that found

with the human urine enzyme may be present. Evidence of isoenzymes of human urine sulphatase A which are inter-convertible has been reported by Stevens *et al* (1973). During $(NH_4)_2SO_4$ reverse gradient solubilization chromatography they observed two peaks of enzyme which were indistinguishable kinetically, antigenically, by gel electrophoresis or isoelectric focusing. The second form of the enzyme was believed to be derived from the first *in vitro* and partial reversion occurred on storage, although the actual chemical difference between the two is unknown.

The data obtained by Nichol and Roy (1965) however, suggests that the difference between the two subunits cannot be explained by such minor changes. When they digested the ox liver enzyme with trypsin and chromatographed the solution they found 36 peptides. The number of peptides which would be predicted, from the number of lysine and arginine residues known to be present in the entire sulphatase A molecule, for non-identical subunits is 49 while the number predicted for two identical subunits is 25. The intermediate value observed suggests that the two subunits are similar and there are probably regions which are identical to both and regions which are unique to each.

These studies will not be complete until it has been determined how similar or dissimilar the two subunits produced in each case are. This could be accomplished by separating them in sufficient quantities to allow peptide mapping of each band. Another approach would be to produce antiserum against the intact enzyme and react it with the two subunits. The degree of cross-reactivity would

indicate how closely related the two are.

Although it has been shown (see Chapter 6) that the modified enzyme has the same molecular weight as the native enzyme and therefore that inactivation is not caused by a break down of the enzyme, the subunit structure may be relevant to its occurrence and to the subsequent activation.

Several cases have been reported where the interaction between the subunits of an enzyme is involved in its regulation. Such a situation has been demonstrated with aspartate transcarbamylase which is composed of two different subunits. One is catalytic and is able to bind the substrate even in the absence of the other. The second is regulatory and binds only effectors (eg. cytosine triphosphate) and through interaction with the first controls the activity of the intact enzyme. Enzymes are also known which have identical active sites on more than one subunit and the binding of a ligand to one can affect the sequential binding of other ligands through interaction between the subunits. An example of this allosteric behaviour is the cooperative binding of nicotine adenine dinucleotide to yeast D-glyceraldehyde-3-phosphate dehydrogenase which has four identical subunits (Kirchner *et al*, 1966). Similar bahaviour is also found with L-phenylalanine ammonia-lyase binding L-phenylalanine (Mouttet *et al*, 1974).

Such mechanisms may be involved in the activation of the substrate-modified sulphatase A which is thought to have two binding sites capable of binding either substrate or activator. If one molecule of each is bound a catalytically

active form of the enzyme is produced whereas if either two substrate or two activator molecules are bound the enzyme remains inactive. The possibility therefore exists that an interaction between the activator and substrate mediated by the enzyme is required for activity.

The hypothesis that the enzyme has two binding sites and the fact that there are two subunits suggests that there may be one binding site per subunit. There is no evidence for this, however, as the subunits have not been isolated.

4. PREPARATION AND SOME PROPERTIES OF SUBSTRATE-MODIFIED SULPHATASE A

4.1 ISOLATION OF SUBSTRATE-MODIFIED SULPHATASE A

4.11 Introduction

Baum and Dodgson (1958) first demonstrated that the substrate-modified form of sulphatase A was stable and could be separated from its substrate and reaction products. Using a crude extract of human liver they were able to obtain, by acetone precipitation from the reaction mixture, a preparation of sulphatase A which contained 80% of the modified enzyme. Since then preparations of the substrate-modified form of purified ox liver (Nicholls and Roy, 1971) and rabbit liver (Lee and Van Etten, 1975a) sulphatase A have been obtained. It was evident, with all of these preparations, that while the modified enzyme was stable enough to be isolated it could also revert back to its native form. For the preparation of substrate-modified enzyme, therefore, conditions of incubation are needed that favour the inactivation reaction and minimize the conversion of the modified enzyme, once produced, back to its native form.

Baum and Dodgson (1958) noted that with the human liver enzyme the rate of inactivation increased at higher pH but that the overall course of the reaction was unchanged and therefore the mechanism of inactivation was the same. Nicholls and Roy (1971) later measured the $t_{\frac{1}{2}}$ for the substrate-induced inactivation of ox liver sulphatase A in the presence of Ba^{2+}, which would eliminate

any possible effect of sulphate. As did Baum and Dodgson, they found the rate of inactivation increased with increasing pH. They measured a $t_{\frac{1}{2}}$ of 5.5 minutes, 2 minutes and 1.2 minutes at pH 5.0, 6.0 and 7.0 respectively. Roy (1978) also observed, with the ox liver enzyme, a sharp increase in the rate of inactivation at pH values less than about 4. He thought, however, that the instability of sulphatase A in dilute solution at low pH contributed to this rate and therefore it would be greater than the actual rate of formation of the substrate-modified enzyme. Between pH 4.5 and 5.5 the rate of inactivation increased, and then dropped again as the pH was raised further. These results differ from those reported earlier (Nicholls and Roy, 1971) where the inactivation rate continued to increase from pH 5.0 to 7.0. The reason for this discrepancy is not known but the experimental methods were very different. A similar peak was found with the initial velocity but the effect was much more marked. At pH 7.0 the initial velocity was approximately 40% of the maximum observed at around pH 5.5 while at pH 7.0 the rate of inactivation was about 60% of its maximum, also observed around pH 5.5.

Roy (1978) further showed that the rate of inactivation decreases if the substrate concentration is decreased or if the ionic strength is increased. Temperature also has an effect, more so than on the initial velocity. Baum *et al* (1958) observed this temperature effect in the time-activity curves produced by the hydrolysis of nitrocatechol sulphate by human liver sulphatase A at different temperatures. Initially the rate

of catalysis increased with temperature but in the later stages the rate of catalysis dropped dramatically at 37.5°C and 50.5°C compared to that observed at 20.5°C and 30.5°C. This was shown not to result from thermal instability of the enzyme and therefore indicates that the increase in the rate of the substrate-induced inactivation is greater than the increase in the catalytic rate as the temperature of incubation is raised. This was also observed by Roy (1978) with the ox liver enzyme. Measurement of the initial velocity and k*, the apparent velocity constant for the inactivation, over the temperature range 24-45°C showed that the initial velocity increased 2.5 fold with this increase in temperature while k* increased 4 fold. Waheed and Van Etten (1980b) also found this relationship with the rabbit liver enzyme. As the temperature was raised from 25 to 50°C the initial velocity increased 7.7 fold but the half-life of the native enzyme decreased by a factor of 180.

As the presence of sulphate also affects the initial stage of the reaction, the concentration of enzyme used in the preparation of the substrate-modified sulphatase A must be kept relatively low. This ensures that the initial velocity is kept low and therefore that the concentration of sulphate produced is not high enough to affect the reaction. Not only does sulphate inhibit the substrate-induced inactivation but Nicholls and Roy (1971) showed that in the presence of substrate, it also displaces the equilibrium between enzyme complexes containing native enzyme and those containing modified enzyme in favour of

the former. This was demonstrated by isolating the enzyme from reaction mixtures at different times and determining the fraction of native enzyme present. They found the percentage of native sulphatase A dropped during the first 7 minutes and then began to rise again until the solution had regained half of the initial activity it had lost. If a lower enzyme concentration was used so that less sulphate was produced or if $BaCl_2$ was added to the reaction the drop in native enzyme was much greater during the initial 10 minutes and no increase was observed during the next 20 minutes. Similar results were obtained by Lee and Van Etten (1975a) with the sulphatase A from rabbit liver. Their modified enzyme preparation was incubated in either 0.01 M nitrocatechol sulphate or 0.01 M nitrocatechol sulphate, 5 mM K_2SO_4 for 20 minutes and then assayed to determine the fraction of native enzyme present. The enzyme incubated in only nitrocatechol sulphate contained 25% native enzyme while that incubated in substrate plus sulphate contained 39% native enzyme. These results suggested that if the degree of reactivation could be minimized by precipitating the sulphate as it was released then the reversion of the modified to the native form of the enzyme would also be reduced.

4.12 Inactivation of Native Enzyme

Taking the above information into consideration, a series of trial preparations were done to determine the optimum conditions for obtaining the substrate-modified form of the enzyme. It was first attempted to incubate sulphatase A in a reaction mixture containing $BaCl_2$ in

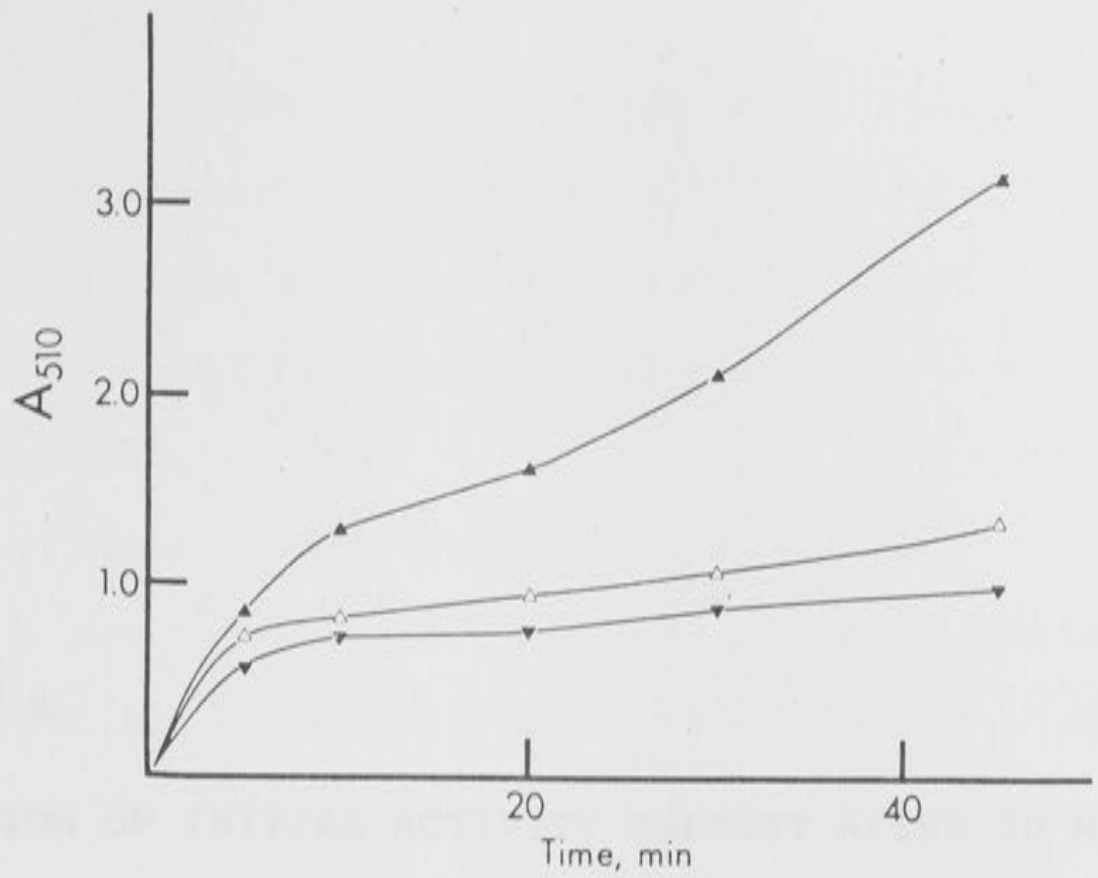
order to eliminate the effect of sulphate.

The incubations were done in 0.5 M sodium acetate buffer, pH 5.6 containing 0.1 M $BaCl_2$ and 1% bovine serum albumin with either 20 mM nitrocatechol sulphate, 40 mM ascorbate 2-sulphate, 20 mM 4-methylumbelliferone sulphate, or 160 mM nitroquinol sulphate as substrate. The reaction was initiated by the addition of sulphatase A and allowed to proceed for 30 minutes at 37°C. The solution was then chilled in ice to stop the reaction and the $BaSO_4$ was removed by centrifugation at 17 000 rpm at 4°C. The precipitate was washed with buffer and the combined supernatants dialyzed and assayed. No activity was found in these solutions either before or after the addition of sulphate indicating that there was no enzyme present. As activity was associated with the precipitate, the enzyme clearly had become adsorbed to the $BaSO_4$. Attempts to reduce adsorption by lowering the salt concentration or by removing the $BaSO_4$ on a Sephadex G-25 column rather than by centrifugation were not fruitful. The barium was therefore eliminated from the inactivation reaction and other conditions sought to give a good yield of the modified enzyme.

Another series of reaction mixtures of 50 mM nitrocatechol sulphate at different pH's and with various concentrations of sulphatase A were incubated at 37°C. The course of the reaction was followed for 45 minutes by taking 0.02 ml aliquots at 5, 10, 20, 30 and 45 minutes and determining the concentration of nitrocatechol present spectrophotometrically. These values were plotted and the

amount of active enzyme remaining could be deduced from the slope of the curve at any given time.

Nitrocatechol sulphate was used as substrate as the apparent rate of inactivation was greater with this substrate than with other substrates such as 4-methylumbelliferone sulphate. The pH was moved away from the optimum pH for hydrolysis of nitrocatechol sulphate (pH 5.6), even though the optimum pH for inactivation also occurs in this region (Roy, 1978), to reduce v_o and therefore the amount of sulphate produced. It was demonstrated in these experiments that a reasonably rapid rate of inactivation occurred at pH's below 8.0 and that the enzyme would also reactivate in this pH range. As mentioned above, the enzyme concentration will affect the amount of modified enzyme in the preparation at the end of the incubation as a consequence of the concentration of sulphate produced in the early stages of the reaction. The maximum enzyme concentration was wanted, therefore, that would not produce sufficient sulphate in the first part of the reaction to affect the later stages. From the data shown in Figure 10 the amount of native enzyme remaining in the preparation at 30 minutes was estimated. These values are given in Table 8.



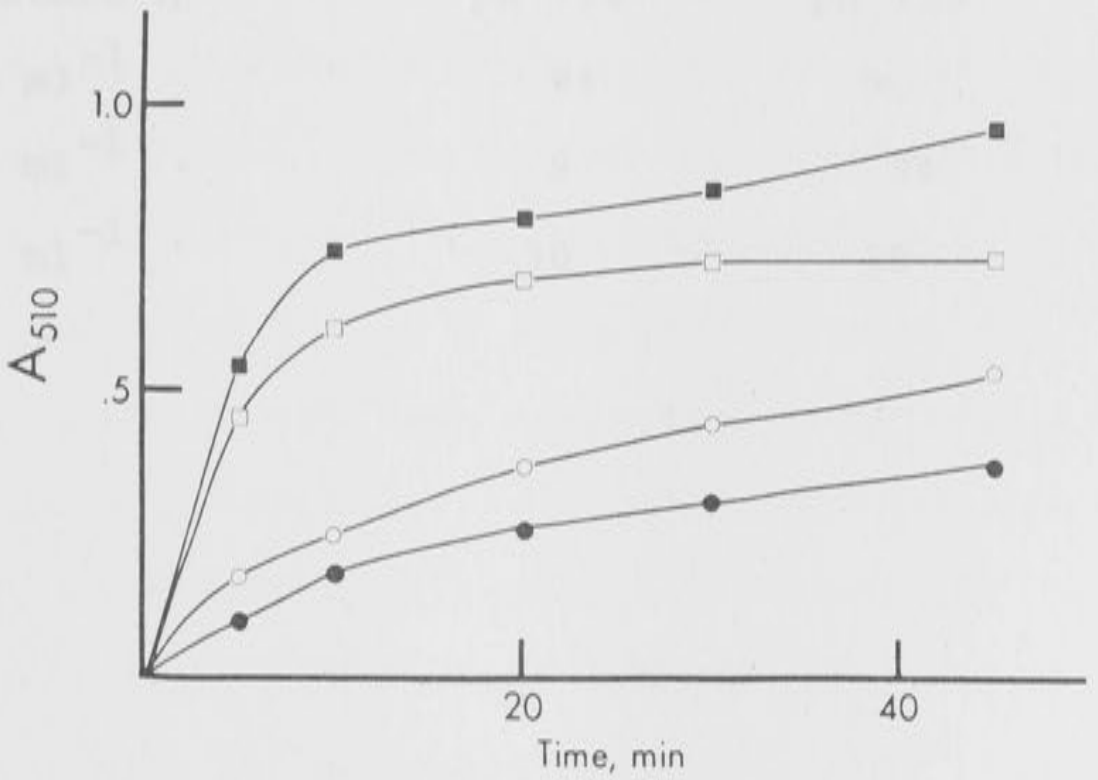


Figure 10 The accumulation of product during 45 minute incubations of native sulphatase A in 50 mM nitrocatechol sulphate at pH 7.0 (triangles), pH 7.5 (squares), and pH 8.0 (circles). The following concentrations of native enzyme were added at zero time: (■, ▲, ●) 25 μg ml^{-1}, (△, □, ○) 20 μg ml^{-1}, (▼) 15 μg ml^{-1}. The concentration of product, nitrocatechol, is shown as the absorbance of the solution at 510 nm after the addition of 0.1 M NaOH.

TABLE 8

FRACTION OF INITIAL ACTIVITY PRESENT AFTER 30 MINUTES INCUBATION IN 50 mM NITROCATECHOL SULPHATE

Sulphatase A	pH 7.0	pH 7.5	pH 8.0
15 µg ml^{-1}	9%		
20 µg ml^{-1}	9	3%	4%
25 µg ml^{-1}	30	25	35

Standard inactivation conditions were taken as pH 7.5, 50 mM nitrocatechol sulphate in 0.1 M Tris-HCl, and 25 µg ml^{-1} sulphatase A incubated for 30 minutes. A preparation routinely containing approximately 10% native enzyme was produced in this way.

4.13 Standard Preparation

In the standard preparation 2.5 mg sulphatase A was incubated for 30 minutes in 100 ml of 50 mM nitrocatechol sulphate, 0.1 M Tris-HCl, pH 7.5 at 37°C and then chilled on ice. All subsequent steps were carried out at 4°C. The enzyme was separated from its substrate and reaction products on a Sephadex G-25 column (2.5 × 81 cm for 100 ml of reaction mixture). The column was prepared and calibrated as given in section 2.6 and eluted with 0.01 M Tris-HCl, 0.15 M NaCl, pH 7.4 at a flow rate of 2 ml min^{-1}. The enzyme fraction was then concentrated by applying to a small (1 × 7 cm) DEAE-Sephadex A-50 column in the above buffer and eluting with 0.05 M Tris-HCl, 0.5 M NaCl, pH 7.4. Fractions were collected (0.5 ml) and assayed for activity with pyrophosphate and nitrocatechol sulphate. The fractions containing enzyme activity were pooled and the absorbance at 280 nm measured. An enzyme solution of approximately 0.6 mg ml^{-1} could be obtained in this way. The final enzyme solution was dialyzed against 5 mM Tris-HCl, pH 7.4, I=0.1 to reduce the concentration of buffer and so minimise the difficulty in recording the early stages of pH-stat assays (see section 2.11).

Table 9 shows the values obtained when aliquots were removed at different stages of the isolation procedure and

assayed. The activity of the solutions was determined in the pH-stat and the extent of modification calculated from initial velocities. No appreciable increase in the amount of native enzyme present occurred during the isolation and routinely this method gave an 80% recovery of enzyme which contained about 90% of the substrate-modified sulphatase A. A typical progress curve obtained with an activation assay in the pH-stat using this preparation is shown in Figure 11.

TABLE 9

AMOUNT OF NATIVE ENZYME IN SUBSTRATE-MODIFIED PREPARATION

Solution Assayed	% native enzyme
Reaction Mixture	4%
Sephadex G-25 Eluate	5%
DEAE-Sephadex A-50 Eluate	5%

4.14 Reduction of Native Enzyme Content

If the above modified enzyme was incubated a second time in 50 mM nitrocatechol sulphate for 30 minutes and reisolated a preparation containing 97% of substrate-modified sulphatase A was obtained. Table 10 shows the increase in modified enzyme produced in two preparations where this was done. As there was no advantage, for most of the work to be described below, in having such highly modified preparations of sulphatase A, this double inactivation, with its accompanying losses of enzyme, was not routinely carried out.

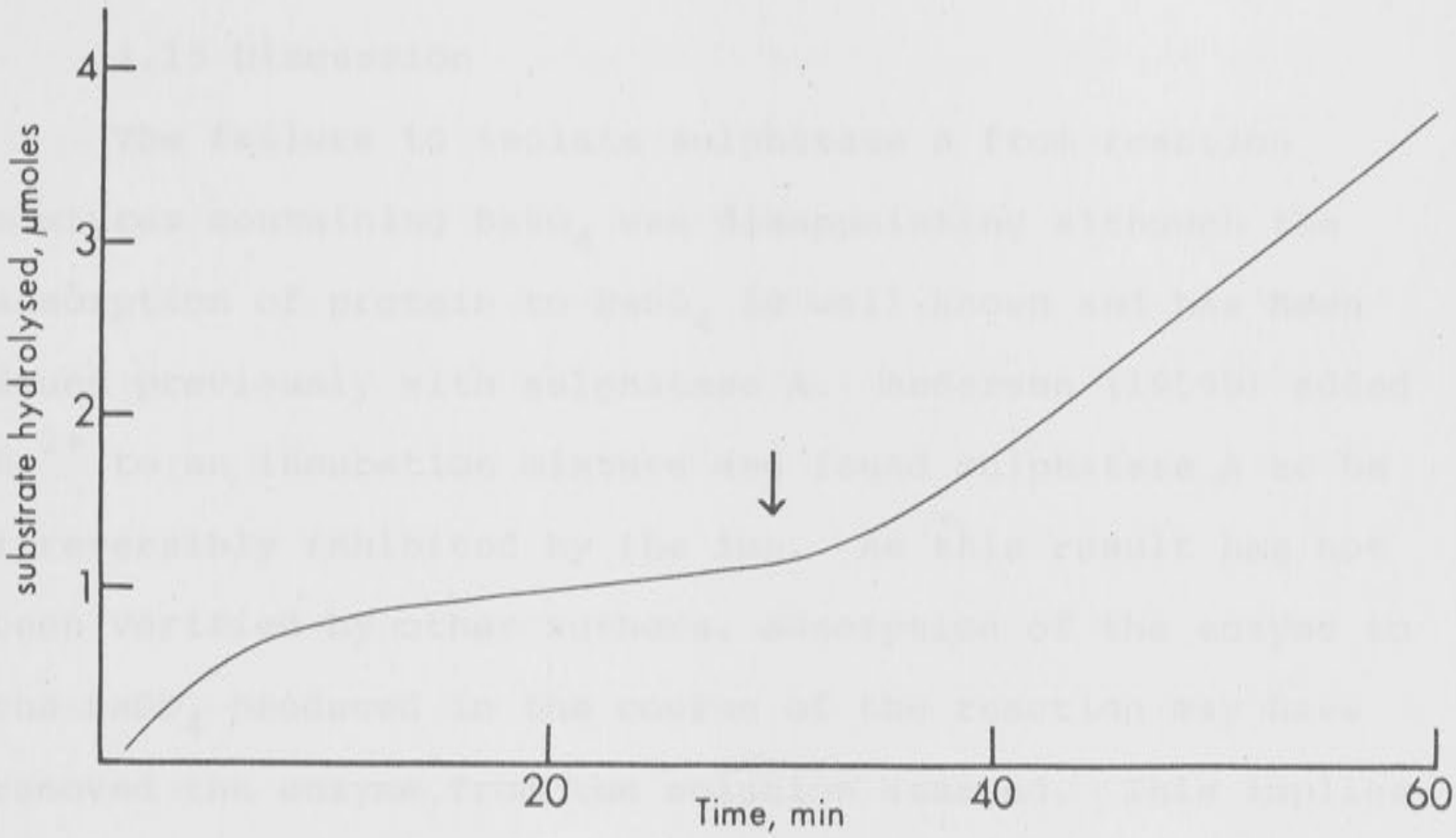


Figure 11 Time course of standard activation assay. Substrate-modified enzyme (8 μg) prepared as described, is added at zero time to 3 mM nitrocatechol sulphate, pH 5.6, 0.1 M KCl. At 30 minutes the solution is made 3 mM SO_4^{2-} and the reaction allowed to proceed for an additional 30 minutes.

TABLE 10

DOUBLE INACTIVATION

	Native Enzyme Content	
First Isolation	15%	12%
Second Isolation	3%	3%

4.15 Discussion

The failure to isolate sulphatase A from reaction mixtures containing $BaSO_4$ was disappointing although the adsorption of protein to $BaSO_4$ is well known and has been found previously with sulphatase A. Andersen (1959b) added Ba^{2+} to an incubation mixture and found sulphatase A to be irreversibly inhibited by the ion. As this result has not been verified by other authors, adsorption of the enzyme to the $BaSO_4$ produced in the course of the reaction may have removed the enzyme from the solution assayed. This implies, however, that the adsorbed enzyme is inactive which has not been shown. Adsorption was also noted by Nicholls and Roy (1971) but they were able to prevent it by adding bovine serum albumin to the solution. This was not found to be an effective remedy under the conditions used in the present preparative reactions: although BSA was present in the incubation mixtures the enzyme was still carried down with the precipitate. Other authors had no problem with this occurring. Waheed and Van Etten (1980b) were able to isolate the rabbit liver enzyme from a solution containing $BaCl_2$ without the addition of BSA. Stinshoff (1972) was also able to assay the enzyme from human kidneys in 1 mM $BaCl_2$ without altering the reaction parameters of the

initial phase of the reaction. No attempt was made in this instance to isolate the enzyme from the incubation mixture and the possibility therefore remains that the enzyme was adsorbed but that this did not affect its activity. This would be consistent with the observations made in these trials in that the $BaSO_4$ precipitate was catalytically active although no attempt was made to study the kinetics of the adsorbed enzyme. The difference between the reaction conditions used in these preparations and those used by these other authors was not obvious but it is known that adsorption can be strongly influenced by seemingly minor differences which affect the way in which the $BaSO_4$ comes out of solution.

It is obvious that, whatever the mechanism of the substrate-induced inactivation, the fraction of enzyme which is modified is dependent on the presence and concentration of substrate and sulphate. The final conditions adopted for the preparation of substrate-modified sulphatase A are similar to those given by Nicholls and Roy (1971) in their method for isolating the modified enzyme from ox liver sulphatase A. They differ only in the pH, and therefore the buffer, used. These authors used 0.1 M sodium acetate buffer, pH 6.0 and added an additional 0.1 M NaCl. Using an enzyme concentration of 1-10 $\mu g\ ml^{-1}$ they obtained a preparation which contained between 10 and 50% native enzyme. The use of the higher pH, in the preparation given here, reduced reactivation and therefore enabled a greater concentration of enzyme to be added. The above conditions differ considerably from

those used by Waheed and Van Etten (1980b) in their isolation of the substrate-modified form of rabbit liver sulphatase A. They added 35 μg ml^{-1} enzyme to a solution of 140 mM nitrocatechol sulphate, 3 mg ml^{-1} $BaCl_2$, pH 6.0 and incubated for 5 minutes at 50°C where the half-time for inactivation is 0.8 minutes (Waheed and Van Etten, 1980b). Separation of the enzyme was accomplished on a Sephadex G-25 column and the solution was then concentrated by dialysis against Aquacide. It is worth noting, however, that at least in the absence of substrate substrate-modified rabbit liver sulphatase A is unstable above 37°C (Waheed and Van Etten, 1980b). An enzyme preparation which retained 18% of its original activity was obtained using these conditions.

The results of incubating the substrate-modified enzyme with nitrocatechol sulphate a second time are interesting as they provide evidence for the hypothesis put forth by Nicholls and Roy (1971) that the native and modified enzyme exist in an equilibrium which is shifted towards the modified form by the presence of substrate. These authors thought that the modified enzyme had two binding sites and when substrate was bound to both of these sites, an inactive complex, FS_2, was produced. The presence of substrate in the absence of sulphate would therefore draw the equilibrium between ES and FS towards the latter by the removal of FS to form FS_2. The effect of other factors on the position of this equilibrium will be discussed in the next section.

4.2 SOME PROPERTIES OF THE PREPARATION OF SUBSTRATE-MODIFIED SULPHATASE A

The general kinetic characteristics of the preparation of substrate-modified sulphatase A are shown in Figure 11. It can be seen in this figure that there are two regions where enzyme activity is observed: during the first 10 minutes and after the addition of sulphate at 30 minutes. It is thought that the substrate-induced modification is not complete under the preparative conditions and that both native and substrate-modified sulphatase A are present in this preparation. The initial velocity is therefore believed to result from the remaining native enzyme and the sulphate-activated velocity from the substrate-modified enzyme. Because only a small fraction of native enzyme is present it too becomes modified during the initial 30 minutes of the reaction (as can be seen by the loss of activity) and at the point of sulphate addition essentially all of the enzyme present will be modified. It should be noted in particular that the ratio method used to calculate the extent of modification (section 2.23) makes these assumptions.

By studying the effect of different modifiers on the initial activity and comparing the observations to those made with the native enzyme, the validity of this assumption can be evaluated. The response of the sulphate-activated velocity to these modifiers helps to characterize the substrate-modified enzyme and their effect on the ratio of initial to activated velocities further defines the relationship between the two forms.

4.21 Inhibition by Sulphite

Although sulphite is known to be a potent inhibitor of the native enzyme very little information has been obtained about its effect on the modified enzyme. It will inhibit native sulphatase A with a Ki of 0.2 μM for the ox liver enzyme (Roy, 1976) and a Ki of 1.1 μM for the rabbit liver (Lee and Van Etten, 1975a) and human liver (James, 1979) enzymes. Waheed and Van Etten (1980b) could detect no inhibition by sulphite of the residual activity in their preparation of the substrate-modified rabbit liver enzyme. This suggests that the residual activity is not due to native enzyme remaining in the preparation but it should be noted that Lee and Van Etten (1975a) assumed in their calculation of the percentage of modified enzyme in their preparations that it was. No explanation is put forward as to why the native enzyme would be inhibited by sulphite in the one instance and not affected in the other.

The effect of sulphite on the activity of the modified enzyme preparation at different stages of the reaction was investigated with the sulphatase A from ox liver. The initial velocity was measured at various sulphite concentrations and the results obtained are given in Table 11. It should be noted that the sulphite concentrations stated may be grossly overestimated due to the instability of the ion even though solutions were made up immediately before use. It was not attempted to accurately define the concentration as only the effect is important in these findings. Protonometric assays were used as they require much less enzyme and reasonable curves could be obtained

even though the enzyme solution being measured was 92% modified. As can be seen, complete inhibition of the residual activity was achieved. While this is not conclusive evidence that the activity is in fact due to native enzyme remaining in the solution it does not create the discrepancy which is found with the rabbit liver enzyme.

TABLE 11

SULPHITE INHIBITION

	v_o (mV min^{-1})	Inhibition
control	2.48 ± 0.05	
0.25 µM SO_3^-	2.23 ± 0.02	10%
0.36 µM SO_3^-	1.96 ± 0.11	21%
1.00 µM SO_3^-	0	100%

The substrate-modified enzyme could also be activated by 0.2 mM SO_3^- (see section 5.42) which means that for the ox liver enzyme no anion has been found which is a competitive inhibitor of the native enzyme and is unable to activate the modified enzyme. In contrast, a preparation of substrate-modified rabbit liver sulphatase A which was shown to be activated by sulphate was not affected by sulphite (Waheed and Van Etten, 1980b). This observation demonstrates that with the rabbit liver enzyme all competitive inhibitors of the native enzyme are not effective activators of the modified enzyme.

4.22 Stability in Dilute Solution

There is no evidence to suggest that substrate-modified sulphatase A is unstable at the concentrations normally

obtained in the standard preparation, that is, at 0.5-0.7 mg ml^{-1}. With more dilute solutions the ability of the enzyme to be activated by sulphate is lost on standing. For example, a solution of modified enzyme of approximately 0.1 mg ml^{-1} assayed after storage at 4°C in 5 mM Tris-HCl, pH 7.4 for 3 weeks gave an initial velocity comparable to that which was measured when it was first prepared but, on the addition of sulphate, activated to only 30% of the activity it had shown before storage. These values are given in Table 12. This loss of activity cannot be reversed by concentrating the enzyme solution. Similar instability of the native enzyme has been observed at pH values less than about 5.6 and at concentrations less than 50 µg ml^{-1} but not at pH 7.5 (Jerfy *et al*, 1976). The modified enzyme therefore appears to be less stable under these conditions than the native form.

TABLE 12

INSTABILITY OF SUBSTRATE-MODIFIED SULPHATASE A IN DILUTE SOLUTION

	Activity	
	v_o	SO_4^{2-} activated
	(µmoles min^{-1} mg^{-1})	(µmoles min^{-1} mg^{-1})
assay at preparation	23	59
assay after 3 weeks at 4°C	25	19

A difference in the stability of the native and substrate-modified enzyme has also been observed with the rabbit liver sulphatase A (Waheed and Van Etten, 1980b). They found that while the native enzyme was stable between 37°C and 50°C the modified enzyme was readily denatured in this temperature range.

4.23 Reversion to Native Enzyme

A. Temperature

The reversion of modified enzyme to its native form when heated in buffer was first shown by Baum and Dodgson (1958) with human liver sulphatase A. They observed that their modified enzyme preparation regained approximately 75% of its initial activity after incubating the solution for 2 hours at 37°C and then allowing it to stand at room temperature for 18 hours. Later, Nicholls and Roy (1971) estimated the $t_{\frac{1}{2}}$ for formation of native from modified enzyme to be 4 hours at pH 6 and pH 8 and 37°C for the ox liver enzyme and Lee and Van Etten give a $t_{\frac{1}{2}}$ of 36 hours at 37°C and pH 4.8 for the rabbit liver enzyme (1975a). A more detailed study of the ox liver sulphatase A was undertaken in order to find suitable storage conditions for the modified enzyme, thus allowing large scale preparations of it to be made. The information is also important when analyzing changes in the kinetic behaviour of the enzyme over long incubation times during assays or other measurements.

Aliquots of 50 µl of the modified enzyme (0.5-0.6 mg ml^{-1}) in either 50 mM Tris-HCl, pH 7.4, I=0.1 or 70 mM sodium acetate buffer, pH 5.0 I=0.1 were sealed in glass

tubes and kept at the appropriate temperature for the required time. At specified times during the incubation single tubes were chilled in ice and their contents assayed in the pH-stat. The amount of native enzyme present in the solution was calculated from the initial velocity. The results are given in Figure 12 for incubations done at pH 7.4 at 20°, 37° and 45°C and at pH 5.0 at 37°C. The values of $t_{\frac{1}{2}}$ given in Table 13 below were determined directly from the graphs.

TABLE 13

THERMAL REVERSION OF SUBSTRATE-MODIFIED TO NATIVE SULPHATASE A

	$t_{\frac{1}{2}}$		
	20°C	37°C	45°C
pH 7.4	60	5	1.5
pH 5.0		30	

The value at pH 7.4 and 37°C agrees with that obtained by Nicholls and Roy for the ox liver enzyme at pH 6 and 8. It will be noted that the reversion is much slower at pH 5.0 than at pH 7.4. It is possible that the polymerization of the enzyme at the lower pH and this enzyme concentration is a contributing factor to the increased stability of the modified form of the enzyme.

The activation energy for this reaction calculated from the $t_{\frac{1}{2}}$ values at pH 7.4 at 20°C and 37°C is 6 kJ $mole^{-1}$. This is very similar to the estimate of 5 kJ $mole^{-1}$ made by Lee and Van Etten (1975a) for the thermal reversion of the modified rabbit liver sulphatase A to its

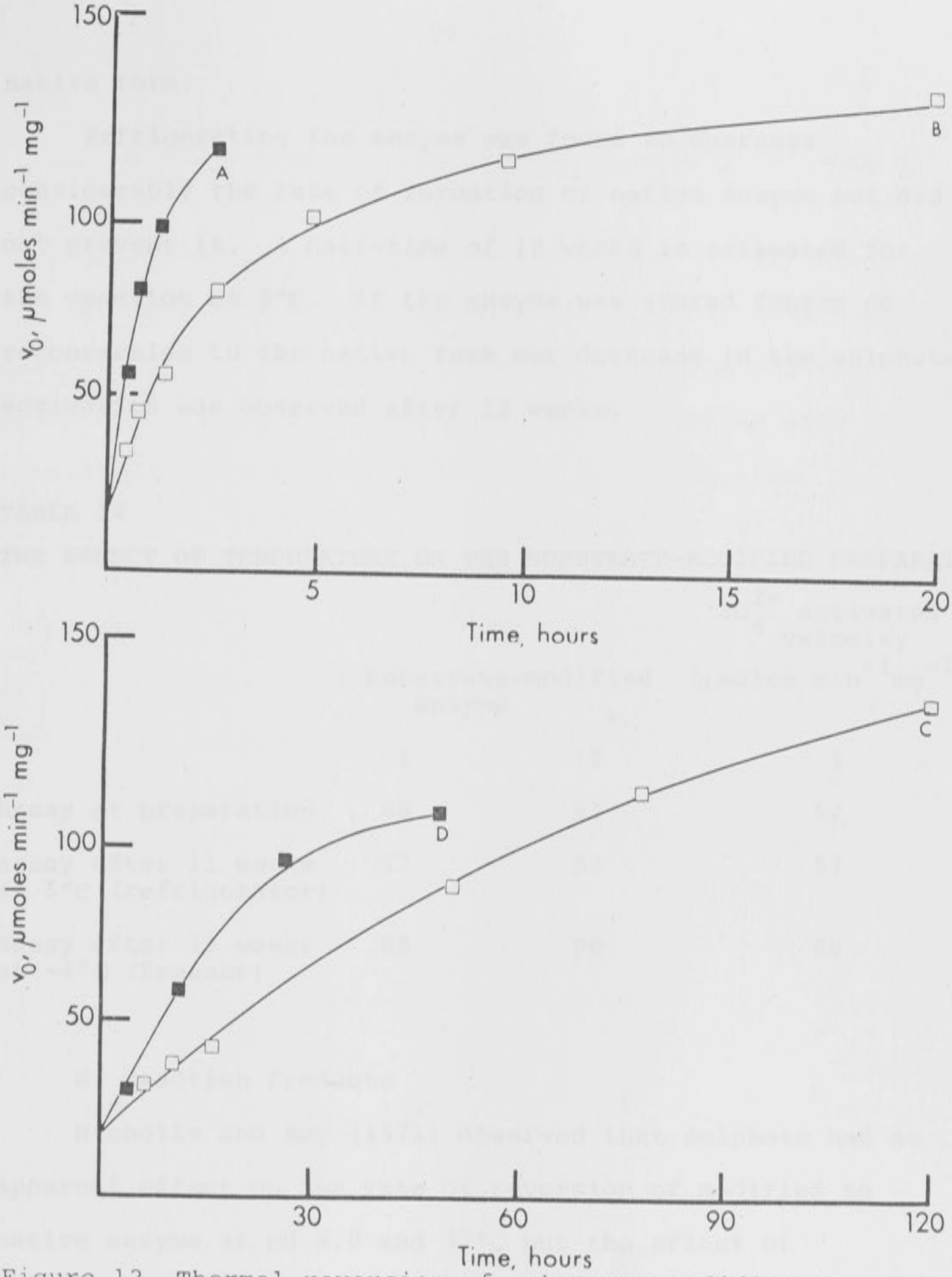


Figure 12 Thermal reversion of substrate-modified to native enzyme. Aliquots of a preparation of substrate-modified enzyme originally containing 8% of native enzyme were incubated under the conditions given and the initial velocity of the solution determined. The enzyme was incubated in 5 mM Tris-HCl, pH 7.4 at 45°C (A), 37°C (B), and 20°C (C) and in 0.07 M sodium acetate buffer, pH 5.0 at 37°C (D).

native form.

Refrigerating the enzyme was found to decrease considerably the rate of formation of native enzyme but did not prevent it. A half-time of 10 weeks is estimated for the reaction at 5°C. If the enzyme was stored frozen no reconversion to the native form nor decrease in the sulphate activation was observed after 12 weeks.

TABLE 14

THE EFFECT OF TEMPERATURE ON THE SUBSTRATE-MODIFIED PREPARATION

	% Substrate-modified Enzyme		SO_4^{2-} activated velocity (μmoles $min^{-1}mg^{-1}$)
	I	II	I
Assay at preparation	88	92	62
Assay after 11 weeks at 5°C (refrigerator)	47	52	57
Assay after 11 weeks at -4°C (freezer)	88	90	60

B. Reaction Products

Nicholls and Roy (1971) observed that sulphate had no apparent effect on the rate of reversion of modified to native enzyme at pH 6.0 and 37°C but the effect of nitrocatechol was not investigated. A series of incubations were therefore done as described above at 37°C and pH 5.6 to determine the effect of the reaction products on the formation of native enzyme in the absence of substrate. The results are given in Table 15. Sulphate had no effect on the rate but 4-nitrocatechol increased it. This increase was not observed when both 4-nitrocatechol and sulphate were

TABLE 15

REVERSION OF SUBSTRATE-MODIFIED TO NATIVE SULPHATASE A

A substrate-modified enzyme preparation originally containing 13% native enzyme was incubated at 37°C in 0.125 mM sodium acetate buffer, pH 5.6, 0.125 mM NaCl with the additions given below. The % native enzyme was calculated from the initial velocity of the solution at the given time.

	Native enzyme (%)	
	3h	6h
buffer	13	15
+ 3 mM K_2SO_4	17	20
+ 3 mM 4-nitrocatechol	24	36
+ 3 mM K_2SO_4 + 3 mM 4-nitrocatechol	18	18

present. These results suggest that the activation of the modified enzyme brought about by 4-nitrocatechol may differ from that caused by sulphate. 4-Nitrocatechol may be affecting the rate at which native enzyme is being reformed whereas sulphate is activating the modified enzyme itself. The manner in which sulphate prevents the nitrocatechol effect is not understood. It may be that sulphate stabilizes the conformation of the substrate-modified enzyme and inhibits the interaction with nitrocatechol. Evidence for such a stabilizing effect was obtained by Waheed and Van Etten (1980b) using rabbit liver sulphatase A. In their studies of the conformation of the substrate-modified enzyme and the activated modified enzyme they found that 5 mM sulphate added to the buffers used in the isolation procedure reduced the loss of secondary structure observed with the isolated activated enzyme.

The effects of sulphate on the substrate-modified enzyme under different conditions will be discussed more fully in Chapter 8.

C. Activation by Sulphate

The change which is reflected by the increase in activity induced by the addition of SO_4^{2-} has not been elucidated. The activation could result from the formation of native enzyme from substrate-modified enzyme but this seems very unlikely in light of the data available. In particular it would not explain the finding of Baum and Dodgson (1958) that the addition of $BaCl_2$ to an assay mixture after activation had occurred resulted in immediate inactivation. The enzyme could be reactivated

by the subsequent addition of an excess of sulphate which is a competitive inhibitor of the native enzyme. If the activated velocity was due to native enzyme it would have been expected to increase when the sulphate was precipitated and decrease when sulphate was added. It is also difficult to explain why the velocity becomes linear if the activity is due to the reformation of native enzyme. Since it is known that the presence of sulphate reduces the rate of substrate-induced inactivation but does not eliminate it, the linear rate would have to represent a steady state where the rate of formation of substrate-modified enzyme equalled the rate of reversion of the modified to the native enzyme. Data presented in section 7.32A indicates that such a dynamic equilibrium cannot be occurring. If it were, the labelled modified enzyme isolated after activation would not still contain 1 mole of ^{35}S per mole of enzyme.

The activity could result from the formation of another stable form of the enzyme. Isolation of the activated enzyme should help to clarify the situation and therefore the modified enzyme, isolated as given above, was activated with sulphate and reisolated 30 minutes after the addition of the activator. The kinetic characteristics of the enzyme species present at that point could then be determined.

The activation was carried out in a large vessel in the pH-stat allowing the course of the reaction to be monitored. Modified enzyme (25 µg) was incubated for 30 minutes in 50 ml of 3 mM nitrocatechol sulphate, pH 5.6, 0.1 M KCl. At 30 minutes 30 µmoles K_2SO_4 was added and

the reaction followed for a further 30 minutes. The vessel was then removed from the pH-stat, put immediately on ice and the enzyme isolated at 4°C as before. A 2.5 × 40 cm column of Sephadex G-25 and a 1.5 × 4 cm column of DEAE-Sephadex A-50 were used with the same buffers as given for the isolation of the modified enzyme. The concentration of enzyme in the final solution was determined fluorometrically and an aliquot assayed in the pH-stat for 60 minutes. A 72% recovery of enzyme was made and as can be seen in Figure 13 the progress curves of the inactive and activated enzymes are similar. The initial velocity of the activated enzyme was greater but the sulphate activated rate was identical to that of the original preparation indicating that the fraction of native enzyme has increased. This increase is in agreement with the results of Nicholls and Roy (1971) who determined the fraction of native enzyme present at different times during the course of the reaction. These results were described in section 4.11 and showed that sulphate in the presence of substrate, as in the assay solution, causes a shift in the equilibrium between the modified and native enzyme toward the latter. The activity observed after the addition of sulphate cannot be due to the observed 30% increase in native enzyme however, as explained above, although it probably does make some contribution as native sulphatase A is only inhibited by about 50% in 3 mM SO_4^{2-}. It also appears that the activity is not due to the formation of a third stable form of the enzyme. This is demonstrated by the similar progress

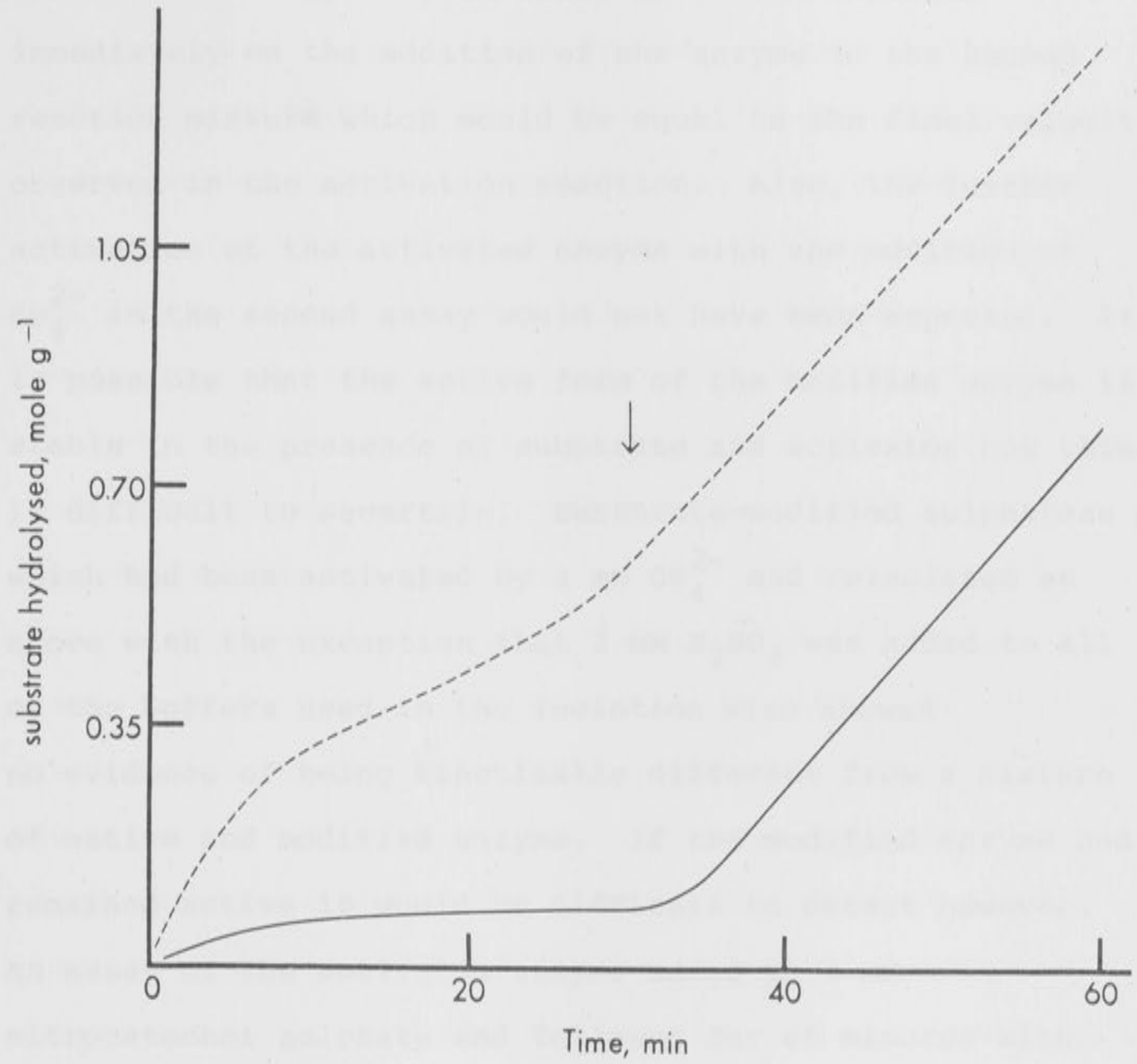


Figure 13 Isolation of activated substrate-modified enzyme. Progress curves for the hydrolysis of 3 mM nitrocatechol sulphate by a standard preparation of substrate-modified sulphatase A (——) and by the same sample which had been activated by SO_4^{2-} and reisolated (----). In both cases 30 µmoles SO_4^{2-} were added to the reaction mixture at 30 minutes.

curves produced by the enzyme both before and after activation. If the activated enzyme had been stable a linear rate of hydrolysis would have been expected immediately on the addition of the enzyme to the second reaction mixture which would be equal to the final velocity observed in the activation reaction. Also, the further activation of the activated enzyme with the addition of SO_4^{2-} in the second assay would not have been expected. It is possible that the active form of the modified enzyme is stable in the presence of substrate and activator but this is difficult to ascertain. Substrate-modified sulphatase A which had been activated by 3 mM SO_4^{2-} and reisolated as above with the exception that 3 mM K_2SO_4 was added to all of the buffers used in the isolation also showed no evidence of being kinetically different from a mixture of native and modified enzyme. If the modified enzyme had remained active it would be difficult to detect however. An assay of the activated enzyme added to 3 mM nitrocatechol sulphate and followed for 60 minutes with the addition of 30 µmoles SO_4^{2-} at 30 minutes produced a progress curve identical to that observed with the activated enzyme isolated without sulphate in the buffer. Since dilution of the sulphate would occur when the reaction was initiated, the inactive enzyme may have been reformed at that point and therefore this does not conclusively show that an active form of the enzyme was not present before dilution. This problem can only be overcome by adding the enzyme directly to a reaction mixture containing substrate and sulphate. Interpretation

of this type of assay is very difficult when the enzyme being assayed contains relatively large fractions of both native and modified sulphatase, as is found with the preparation of the activated enzyme, because both forms contribute to the initial velocity. If essentially all of the enzyme present is modified, as in a preparation of substrate-modified enzyme which has been incubated twice (section 4.14), then a progress curve comparable to that observed in the standard activation assay after the addition of SO_4^{2-} is observed. There is a gradual increase in activity until a linear velocity is reached. If substantial amounts of native enzyme are present a hyperbolic progress curve is also present in the initial stages because the native sulphatase A will only be about 50% inhibited under these conditions. The sum of these two activities produces a progress curve which is slightly convex downwards during the very early stages of the reaction and again becomes linear. This is observed both with the original modified preparation and that which had been activated and reisolated in the presence of SO_4^{2-}. It is not possible to determine whether or not the component due to the modified enzyme in the early stage is linear.

The presence of activating anions therefore appears to generate an active state in the modified enzyme which is unstable due either to an instability in the absence of substrate and activator or to the inactive enzyme being reformed with the release of the products of the catalytic reaction.

4.24 Discussion

As was mentioned above, it is thought that an equilibrium exists between the native and substrate-modified forms of sulphatase A which is affected by the relative concentrations of substrate and sulphate. As is seen in section 4.23 A, there is a temperature-dependent reversion of the modified to the native enzyme in the absence of both these compounds. If substrate alone is present, this reaction is inhibited as is shown by the fact that the substrate-modified enzyme preparation will lose its residual native activity when added to an assay mixture and remain inactive for many hours if no sulphate is added. It is still able to activate, however, if an appropriate anion is added (Nicholls and Roy, 1971; Prosser, unpublished results). The substrate therefore appears to shift the equilibrium towards the formation of modified enzyme and prevent the thermal reversion to the native form. The latter may simply reflect the continued inactivation of the native enzyme in the presence of substrate rather than inhibition of the reversion. That is, the rate of inactivation of any native enzyme present may more than compensate for the rate at which it is being reformed from the modified enzyme. It is also possible that with the formation of a modified enzyme complex containing two bound substrate molecules, the reversion to native enzyme is inhibited because FS_2 cannot convert directly to ES_2 and therefore must first lose at least one substrate molecule.

Sulphate alone has no apparent effect on this equilibrium but does seem to cause a shift toward the native enzyme if substrate is also present. Nicholls and Roy (1971) suggest that sulphate influences the position of the equilibrium by binding preferentially to E and forming larger concentrations of EI compared to FI and FI_2. If this is the case, it is difficult to see why sulphate has no effect in the absence of substrate. It seems more likely that it is the activation process itself which leads to the increase in native enzyme content. It is possible that the probability of the modified enzyme reverting back to its native form is increased at some point as the activated modified enzyme complex, FIS, breaks down to release the reaction products (and sulphate?).

5. KINETICS

5.1 INTRODUCTION

The most sensitive criterion for assessing changes in an enzyme's structure or interactions with other molecules is through a change in its catalytic activity. This is a reflection both of the minute amounts of enzyme which can be detected kinetically compared to the quantities required for physical determinations, and, of the dependency of an enzyme's activity on its immediate environment and the integrity of the enzyme molecule. The rate of reactions catalyzed by nanogram quantities of enzymes can be accurately measured using radioactively labelled or fluorometric substrates whereas milligrams of protein are required for most physical measurements. The large size of most enzymes also means that only major changes in structure or conformation are likely to be detected directly while very small differences may affect to a measurable extent the specific activity or the enzyme's response to an effector. For instance, changes in structure due to the binding of ligands, to changes in temperature or to interaction with solvents of different compositions can be observed through differences in the kinetic constants for the reaction under the appropriate conditions. Kinetic studies also have the advantage that, from information about the response of an enzyme to variations in the conditions of assay, predictions can be made regarding rate determining steps and interactions between substrates, inhibitors, and activators which aid in the understanding of the catalytic and control mechanisms of the enzyme.

The substrate-modified form of ox liver sulphatase A was first detected kinetically, as discussed previously, and its altered activity remains the only way in which it can be distinguished from the native enzyme. It is essential to fully characterize the kinetics of the modified enzyme both in order to define the differences between its behaviour and that of the native enzyme and to try to determine a mechanism to account for the observed inactivation and subsequent activation. It is also necessary to examine the kinetics of any preparation of the modified enzyme to ensure that the observed loss of activity represents the formation of substrate-modified enzyme and does not result from any other cause. This is particularly important when isolating the enzyme from a reaction mixture as it is possible for the enzyme to become partially denatured or otherwise altered during the isolation procedure. Thus, apart from the value of the information obtained in earlier work about the substrate-modified enzyme without its preliminary separation from the reaction mixture, it is important to be familiar with these kinetic data for practical reasons.

Several kinetic studies have been made with sulphatase A from different sources which investigated both the inactivation and activation processes. Most of these were carried out by adding native enzyme to the reaction mixture and following the entire course of the reaction through the formation of the substrate-modified enzyme and its subsequent activation, with appropriate additions of reagents at different times during the incubation.

From this type of study it was shown that the sulphatases A from all sources tested lose their activity when hydrolysing any of a variety of aryl sulphates which are used as experimental substrates. This inactivation is also observed when compounds such as ascorbate 2-sulphate and glucose 3-sulphate are hydrolysed. It was demonstrated that when nitrocatechol sulphate is used as substrate the products of the reaction, particularly sulphate, can reactivate the enzyme. The addition of other anions such as phosphate, pyrophosphate, sulphite and selenate similarly induce activation. The other reaction product, nitrocatechol, also has this effect but to a much lesser degree and possibly through a different mechanism as was discussed in section 4.23 B.

Attention was first directed by Dodgson and Spencer (1956b) towards the inorganic sulphate as being the major factor in the observed spontaneous reactivation of sulphatase A. Baum and Dodgson (1958) later demonstrated beyond doubt the effect of this anion on the enzyme activity. They allowed a sample of the human liver sulphatase A to inactivate and reactivate and then added barium chloride to the reaction mixture to precipitate the sulphate which had been produced during the reaction. This resulted in a total loss of activity. When an excess of sulphate was added to the solution the activity was restored showing that the accumulation of sulphate was responsible for the increase in activity. They also noted that the activation step was inhibited by both substrate and sulphate if their concentrations were raised above optimum levels. This was

instrumental to the development of the mechanism which they proposed and remains convincing evidence for the presence of two binding sites on the substrate-modified enzyme.

When the substrate-modified enzyme was isolated and shown to be stable enough to store as such, more precise data could be obtained. This is largely due to the fact that it allows the composition of the enzyme preparation to be determined. That is, the amount of native enzyme remaining in the enzyme solution can be measured accurately. This is advantageous as the ratio of native to modified enzyme can be easily altered, as shown earlier, and because the native and modified forms cannot be separated it is essential to know the exact fraction of modified enzyme present in the solution under consideration. With this information one can better ensure that assays are comparable and done under identical conditions. It also eliminates the possibility that an observed difference in enzymatic response is due to differences in the initial enzyme concentration. Isolation of the enzyme from its reaction mixture also provides means of accurately determining the effect of different environments on the equilibrium between the two forms and on the conversion rates from one to the other.

Baum and Dodgson (1958) did separate the substrate-modified human liver enzyme from the substrate and products present in the solution but did not use this enzyme preparation for any kinetic studies. The separation was accomplished by acetone precipitation and the inactivated enzyme obtained was shown to be stable but not irreversibly altered as it could revert to its original form.

Nicholls and Roy (1971) isolated substrate-modified ox liver sulphatase A from a reaction mixture containing nitrocatechol sulphate. They used this preparation to make more detailed investigations of the effects of varying substrate and sulphate concentrations on the activation of the enzyme and on the equilibrium between the native and modified forms. When the enzyme was assayed with 1 mM nitrocatechol sulphate they observed maximum activation with 1 mM sulphate. They also studied the effect of relatively high concentrations of substrate and sulphate on the activation of the modified enzyme and demonstrated that the concentration of sulphate which caused inhibition was dependent on the concentration of substrate and vice versa.

More recently Lee and Van Etten (1975a) isolated substrate-modified rabbit liver sulphatase A and used this preparation to study the effect of temperature and sulphate on the rate of reversion to the native form. No studies have been done on the activation of the modified form of this enzyme although considerable information has been gathered regarding differences between it and the native enzyme and on the effect of temperature, pH, and other factors on the inactivation (Lee and Van Etten, 1975a; Waheed and Van Etten, 1979, 1980b; Rybarska-Stylinska and Van Etten, 1979).

It is of interest that the ox liver enzyme will undergo substrate modification at lower pH's but cannot be activated by sulphate unless the pH is raised (Roy, 1980). If nitrocatechol sulphate is used as substrate at pH 5.6, the pH at which v_o is maximal, the characteristic loss of

activity is observed and subsequent activation can be induced by the addition of sulphate. In contrast, if the reaction is carried out at pH 4.5 the loss of activity is observed but the addition of sulphate does not lead to activation. The enzyme inactivated at pH 4.5 will activate if the pH is increased to 5.6 before the addition of sulphate and similarly the enzyme inactivated at pH 5.6 will not activate if the pH is dropped to 4.5. This indicates that the inactivation is the same at either pH but that activation does not occur at the lower pH. In contrast to this, Waheed and Van Etten (1979) showed that the substrate-modified form of the rabbit liver enzyme was produced at pH 7.5 but not at pH 4.5. It was initially proposed that this difference resulted from the polymerization of the enzyme, since it exists as a monomer at pH 7.5 and a dimer at pH 4.5, but it was later shown to be purely a pH effect as an antibody-enzyme complex, which was not able to polymerize, showed the same kinetic behaviour (Rybarska-Stylinska and Van Etten, 1979). This agrees with the observations of Jerfy *et al*, (1976) and Roy (1978) that the ox liver sulphatase A monomer and tetramer have identical kinetic characteristics.

Of course, if only the rate of inactivation is of interest then there is no advantage in trying to isolate the modified enzyme from the reaction mixture. Such a study was carried out by Roy (1978) on the inactivation of ox liver sulphatase A. The data obtained from this work must be considered in the present problem as, through the constraints it places on the inactivation process, it provides the

preliminary information about what the differences between the inactive and active forms of the enzyme might be. An apparent velocity constant, k*, was measured using nine different aryl sulphates as substrate, over a range of different concentrations. From these data a value of k, a velocity constant at infinite substrate concentration, for each aryl sulphate was obtained by extrapolation. In all cases this value was approximately 0.23 min^{-1} and therefore it appears to be independent of the initial velocity of the catalytic reaction since the latter varies considerably with the different substrates. This suggests that inactivation proceeds through an enzyme-sulphate complex rather than directly from an enzyme-substrate complex as had been previously thought (Andersen, 1959c; Baum and Dodgson, 1958; Stinshoff, 1972; Lee and Van Etten, 1975a). The sulphate in this complex would, however, be derived from the substrate. As is described in Chapter 7, when nitrocatechol [^{35}S]sulphate was used as substrate the ^{35}S became bound to the substrate-modified enzyme while neither nitrocatechol nor nitrocatechol sulphate could be detected in it. These different data are therefore consistent with each other.

The observations made in the above study by Roy, and by others regarding the behaviour of the immobilized native enzyme are also of interest. Roy (1978) demonstrated that native ox liver sulphatase A bound to CNBr-Sepharose inactivates at a slower rate (i.e. with a k of 0.14 min^{-1} compared to 0.23 min^{-1}) but clearly still inactivates. He also found that the enzyme bound to quartz (unpublished results) inactivated and could be activated by sulphate in

the same manner as the free enzyme. Waheed and Van Etten have shown that rabbit liver sulphatase A bound to Sepharose-sulphatase A inactivates and reactivates at rates comparable to those observed with the free enzyme (1980a). The enzyme from sheep brain was bound to Con-A-Sepharose by Bishayee and Bachhawat (1974) and also found to have the same catalytic properties as the free enzyme. It should be noted that binding to each of these materials involves different interactions with the enzyme. Binding to CNBr-Sepharose is thought to involve mainly the ε-amino groups of lysine and the α-amino groups of the N-terminal amino acid residues while quartz probably adsorbs the enzyme through interaction with the electropositive protein groups, mainly NH_3^+. Concanavalin A, in contrast, would interact with the carbohydrate moieties of the enzyme, with perhaps some protein-protein interaction. Thus it has been demonstrated that the bound enzyme, which would have less conformational freedom than the enzyme in solution, behaves similarly to the free enzyme. It has also been shown that restrictions placed on the mobility of these different portions of the molecule and on their ability to react further did not alter the characteristic kinetics of the enzyme. These findings must be taken into account when considering what the inactivation process actually involves. The fact that the anomalous kinetics were not affected in any of the above cases indicates that gross conformational changes involving a large portion of the protein molecule are probably not involved. It would seem that changes are confined to small alterations in structure, most probably

at or in close proximity to the active site. If the mechanism does involve conformational movement the decrease in the rate of inactivation measured with the Sepharose-bound ox liver enzyme could be explained if conformational changes were hindered in the bound enzyme. It is possible that the active conformation is stabilized or enhanced by restrictions on the movement of the molecule and on the environment surrounding the protein in the Sepharose complex. It is difficult to evaluate such hypotheses without more information about the inactivation mechanism or the differences between the two forms of enzyme.

All of the kinetic work described here involves the activation of the substrate-modified enzyme. Previously very little data had been recorded regarding the activation step and it was hoped that such information, together with what was known about the kinetics of the native enzyme and the inactivation step, would indicate a mechanism of action for sulphatase A acting as an arylsulphatase. The most intriguing characteristic of this stage of the kinetics is the length of time required after the addition of an activator for the modified enzyme to activate. This suggests that the reaction is more complex than the activation observed when the enzyme's response is effectively instantaneous on addition of the activator and where special techniques are required to see the transient phase. For example, the addition of $MnCl_2$ to an assay mixture of 3 mM nitrocatechol sulphate, 0.5 mM acetate buffer pH 4.5 will increase the activity of the native enzyme 2.3 times. This difference is observed in the initial velocity and

there is no alteration to the shape of the progress curve produced in the salt's presence, (Jerfy and Roy, 1973). Similarly the manganese carboxypeptidase A complex is activated by carbobenzoxyglycine with no observable time lag unless NMR relaxation techniques are used (Kushnir and Navon, 1975).

The time required for the activation of the modified enzyme is easily observed using common assay techniques, the velocity taking roughly 10 to 20 minutes to become constant under the usual assay conditions. This time, however, varies with the substrate used and its concentration as well as the activator concentration, and longer times have been observed. The time span is quite long when compared to the estimates of time scales obtained with other proteins for the response to different chemical and physical perturbations. For example, local conformational motions in proteins associated with the binding of small ligands or changes in pressure were found to occur in the region of 10^{-9} sec by Lakowicz and Weber (1973) using fluorescence decay and ^{13}C-NMR techniques. The monomolecular isomerization of glyceraldehyde-3-phosphate dehydrogenase initiated by the binding of NAD was shown to have a rate constant in the order of sec^{-1} (Kirschner *et al*, 1966), whereas, conformational changes observed to occur on the same time scale as the activation of the substrate-modified sulphatase A involve either gross unfolding or disulfide bond formation (Simpson and Kauzmann, 1953).

Similar lag times have, however, been observed with other enzymes. An example would be the activation of

glycogen synthase I by glycogen. This reaction requires 10 minutes to 1 hour to attain a linear rate of reaction depending on the conditions of assay. In this case the lag is believed to be due to a slow binding step rather than any conformational change in the enzyme (Solling and Esmann, 1977). With penicillinase acting with quinacillin (a semisynthetic penicillin) as substrate the time lag observed for changes in activity to occur is thought to be the result of a slow isomerization of the enzyme between two conformational states of different activity (Virden *et al*, 1978). In this instance the inactivation process has a half-time of 30 seconds and the reactivation a half-time of approximately 200 seconds both of which are long compared to that for the turnover of quinacillin in the slow phase ($t_{\frac{1}{2}} \sim$ 2 sec). A third example is the activation of phosphoribosylpyrophosphate synthetase by ATP, Pi and Mg^{++} which has been shown to be the result of a polymerization of the enzyme in the presence of these compounds into polymers containing 16 and 32 subunits. These polymers are catalytically active whereas smaller ones for example, the tetramer or octamer, are not. Five to 10 minutes are required after the addition of these effectors for the maximum activity to be observed (Meyer and Becker, 1977).

The determination of the difference between the inactive and active forms of the substrate-modified sulphatase A is hindered by the fact that the active form has not been isolated. It appears to be unstable as when the substrate and sulphate are removed from the solution only the inactive and native forms are found. The

reactivation has therefore only been studied kinetically. The fact that preincubation with sulphate does not alter the time required to reach a linear velocity indicates that, unlike the reaction with glycogen synthetase, it is not an initial slow binding of the activator that produces the time lag. The possibility remains however that in this case FA_2 is being formed and it is a slow break down of FA_2 to form FA which is responsible for the lag time. This will be discussed below. It is unlikely that a polymerization of the enzyme is involved as, over the enzyme concentration range used, the time lag and linear velocities observed are independent of enzyme concentration. The differences in concentrations used would be less than 10 fold, however, limiting the validity of this conclusion. Other evidence, obtained by comparing the kinetics of the monomer and tetramer, and the molecular weight and association-dissociation pattern of the native and modified enzymes supports the conclusion that polymerization is not involved.

5.2 REACTION SCHEME

Because of the time lag in the activation and the complexity of the reaction, the kinetic study of the substrate-modified enzyme is treated in two sections: the period of time where the rate of hydrolysis is increasing and that in which a linear hydrolysis rate is observed. In order to simplify the nomenclature in this chapter that originally proposed by Nicholls and Roy (1971) has been used which refers to the native form of the enzyme as E and the substrate-modified form as F. In separating the activation stage of the kinetics into these two parts it is considered

that the modified enzyme, as isolated, is inactive but that an active form is generated in the presence of substrate and an activating anion. This active form may simply be the FSI complex in which case all that is required for the activation is the binding of these two ligands and the slow rate of activation would be explained by the slow binding of one or both of them. As evidence for such slow binding has not been obtained with this enzyme it is also possible that another step is required before the complex is catalytically active and therefore a step $F_iSI \rightarrow F_aSI$ is included in the scheme. This step may or may not be rate limiting and, if not, may be relatively unimportant in the overall activation mechanism. Because the enzyme isolated after sulphate activation of the substrate-modified enzyme was kinetically identical to the modified enzyme before activation (see section 4.23 C) and was not a stable third form of sulphatase A, the activity observed after the addition of sulphate is due to an active form of the modified enzyme. For this reason the active complex is written with the enzyme still being modified (F) but being active at this stage, hence F_aSI. As discussed later in this chapter, in order for a steady state velocity to be reached either the activator must be released at some stage during the hydrolysis of substrate so that F_i is reformed or the activator must remain bound to F to give a third, catalytically active form of enzyme which, although it cannot be isolated, may be stable in the presence of reactants. It is thought that the first case is more likely and therefore the modified enzyme is shown regenerated as F_i at the end of the reaction in the

following scheme:

$$F_i + S \rightleftharpoons F_iS \rightleftharpoons F_iSI \rightleftharpoons F_aSI \xrightarrow{k_2} F_i + P + I$$

$$F_i + I \rightleftharpoons F_iI \rightleftharpoons F_iSI$$

k_1 = rate at which modified enzyme is activated

k_2 = rate at which product is released

S = substrate

I = activator

P = products

As can be seen this reaction scheme makes no attempt to define what is occurring during the formation of F_aSI nor what the rate determining step is. It could be either a slow binding of one ligand or a conformational change taking place after the substrate and/or activator are bound. More information is needed before the mechanism will be clear. The scheme is, however, consistent with the information available and the main point is that the extent of activation is dependent on the position of the equilibrium between F_i and F_aSI whatever intermediary steps may be involved in this stage.

5.3 STEADY STATE KINETICS

Although no information about specific rate constants can be obtained from the study of steady-state kinetics, the parameters derived are useful in that they can given general empirical constants. These constants will reflect the entire reaction by combining all of the individual rate

constants into one overall value. Such data, therefore, provides limits within which any proposed mechanism must be confined. Under favourable conditions this approach can also give information about the reaction sequence and estimates for the lower limits of rates. The method is, however, limited by the fact that the equations for complex enzyme mechanisms are so complicated that the parameters cannot be determined from experimental data. Differences between some models are so slight at the level of observation that they cannot be differentiated even with data calculated from the derived equations. Problems thus arise from both restrictions in the accuracy of the data which can be obtained experimentally, and from the fact that several models of complex enzyme mechanisms may be fitted theoretically to any given time course of an enzyme reaction and the linearized or integrated plots derived from it. This is due to the multitude of rate constants and equilibria defined in complex models which leads to many possible combinations of rate-limiting steps and dead end complexes. This ambiguity can only be reduced by simplifying the model mechanism and this of course does not produce the same information.

Despite these limitations the use of the steady state approach when considering the linear portion of the activation curve is preferable to the use of binding equations. Such equations, derived to describe ligand-saturation curves, assume that all forms of the enzyme have the same activity and that the release of product

is the rate determining step. As Frieden (1970) pointed out, with many enzymes, especially those whose activity is regulated, these assumptions are not applicable. With the activation of substrate-modified sulphatase A all forms of the enzyme clearly do not have the same activity and the measurable time lag before a linear rate is observed suggests that there is not a rapid equilibration of all species prior to product release. The rate determining step may therefore involve the conversion of the inactive to the active form of the enzyme and not the release of product. It is shown below that the assumptions required for the use of steady state equations are applicable to the activation reaction and therefore that its use is valid.

5.31 Assay Conditions

The standard substrate-modified enzyme preparation described in Chapter 4 was used in all of these assays. It was not necessary to make any corrections for varying amounts of residual native activity in the different preparations as the enzyme was incubated for 30 minutes in the reaction mixture prior to the addition of sulphate. This resulted in a negligible amount of activity remaining before activation began and it could therefore be assumed that only substrate-modified enzyme was present at this stage. The assays were done in the pH-stat with constant recording which meant the decrease in activity could be easily monitored.

Sulphate was used in most cases as activator to eliminate the need to remove the sulphate necessarily produced by the hydrolysis of the substrate. If this were

not done the problem would arise of having a known activator contaminating the assay mixture even if present in only small quantities compared to that of the added activator. The only convenient way to remove the sulphate produced during the reaction is by adding Ba^{2+} to the incubation mixture. As discussed earlier, adsorption of the enzyme to the $BaSO_4$ could not be prevented in these assays when $BaCl_2$ was present although other authors have been able to overcome this problem. Specifically, Nicholls and Roy (1971) were able to separate the activation due to nitrocatechol from that due to sulphate by precipitating the sulphate in this manner. Nitrocatechol is a much less effective activator however, and all of the other commonly used activators (e.g. phosphate, pyrophosphate, selenate) also react with Ba^{2+}. It is therefore not possible to precipitate the sulphate with Ba^{2+} and study the activation induced by another anion.

Nitrocatechol sulphate was used as substrate throughout and all assays were done at pH 5.6 and 37°C. The modified enzyme was also shown to be activated by sulphate with p-nitrophenyl sulphate as substrate at pH 6.1 and with 4-methylumbelliferone sulphate as substrate at pH 5.6. In these cases however, a linear rate was not observed within 30 minutes after the addition of sulphate and therefore a steady state had not yet been reached. Because of this increase in the time required for the velocity to become linear these substrates were not used routinely but several assays were done using p-nitrophenyl sulphate to demonstrate that the pattern of activation which emerged with nitrocatechol sulphate was not specific to that substrate.

5.32 Results

A. Different Substrates.

Figures 14 and 15 show the dependence of the final velocity on the concentration of substrate and sulphate. As can be seen, there appears to be a correlation between the activator concentration which produces the maximum activation and the concentration of substrate. This pattern was also observed by Nicholls and Roy (1971) with ox liver sulphatase A and is not unexpected as the maximum velocity observed is restricted by the point at which substrate and sulphate inhibition begin. If the hypothesis that the modified enzyme has two binding sites (Baum and Dodgson, 1958; Nicholls and Roy, 1971 and Stinshoff, 1972) is valid and if both sites are able to bind either substrate or activator then the inhibition observed at high substrate as well as high activator concentrations can be explained. If only the FSI complex is active, while both FS_2 and FI_2 complexes are inactive, then once the binding of either substrate or activator begins to dominate the other inhibition will be observed. It is demonstrated in Appendix 1 that this relationship between activity and ligand concentrations is found in a simple model system. Calculation of the concentration of FSI as a function of the concentrations of S and I produces a pattern similar to that of the enzyme activity at various concentrations of nitrocatechol sulphate and sulphate.

It should be noted that the same general pattern is found with other substrates but the optimum concentrations are closely correlated only with nitrocatechol sulphate. This suggests there is a similarity in the binding

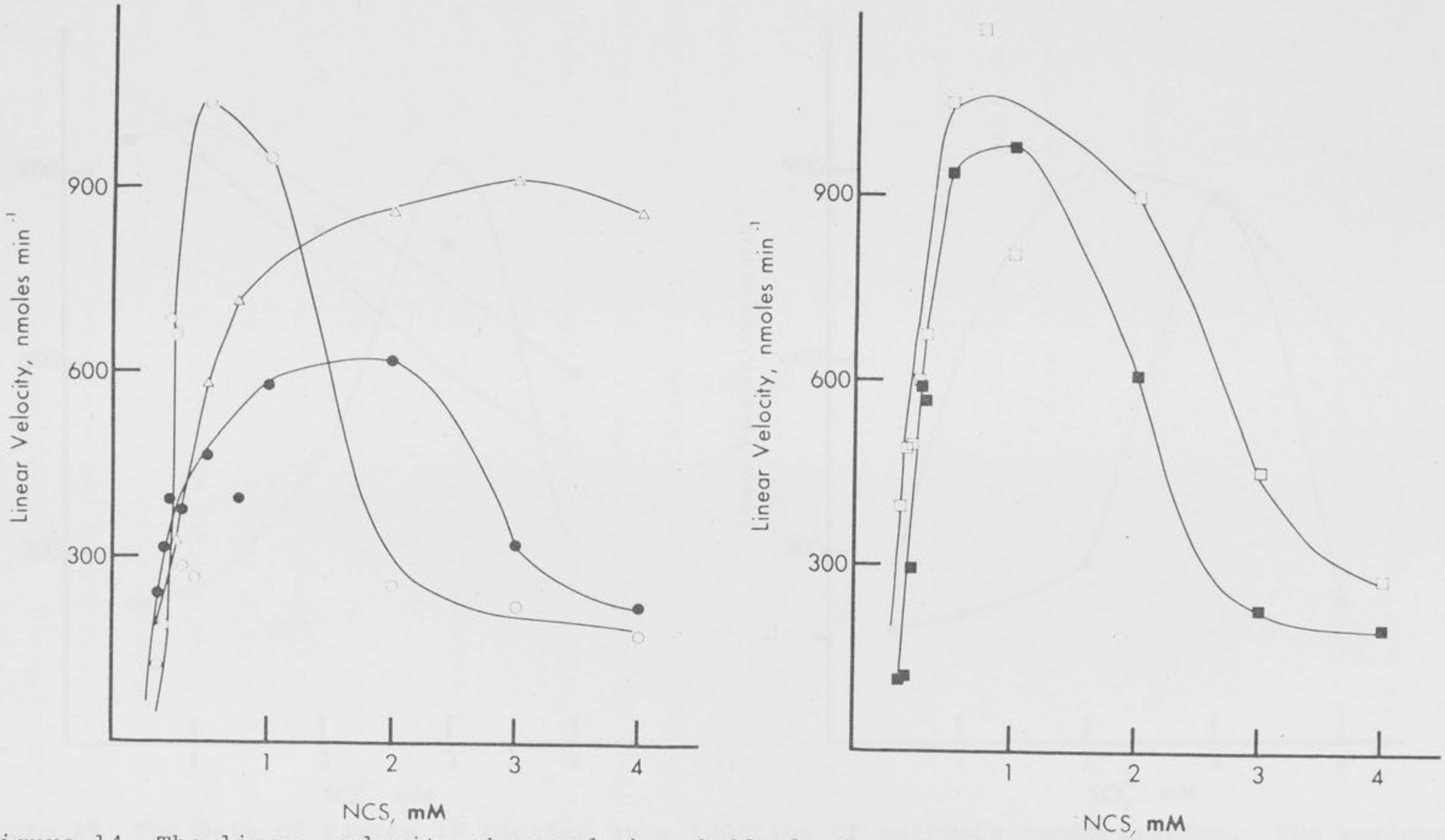
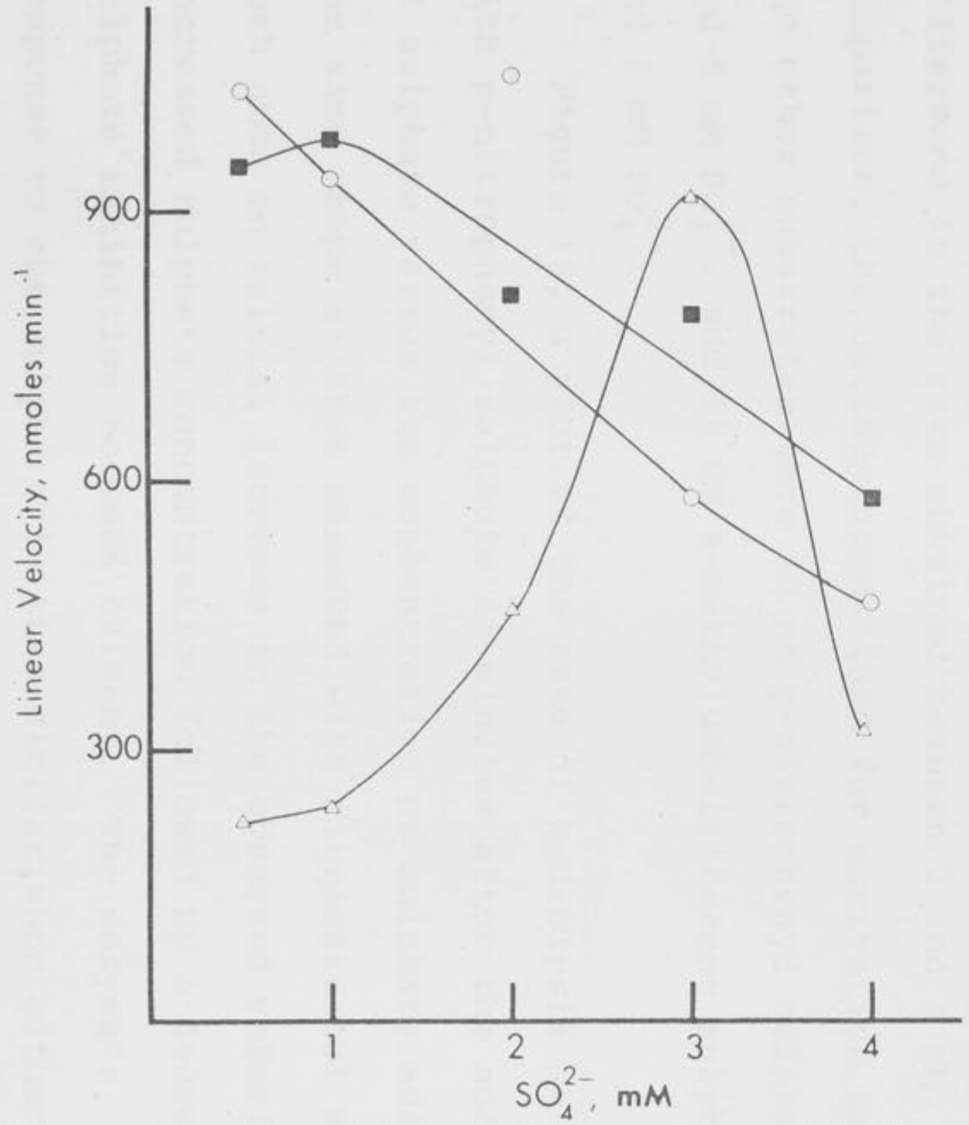


Figure 14 The linear velocity observed when 0.02 ml of a standard preparation of substrate-modified sulphatase A was assayed with nitrocatechol sulphate, pH 5.6, 0.1 M KCl, and the following sulphate concentrations: 0.5 (○), 1.0 (■), 2.0 (□), 3.0 (△), and 4.0 (●) mM.



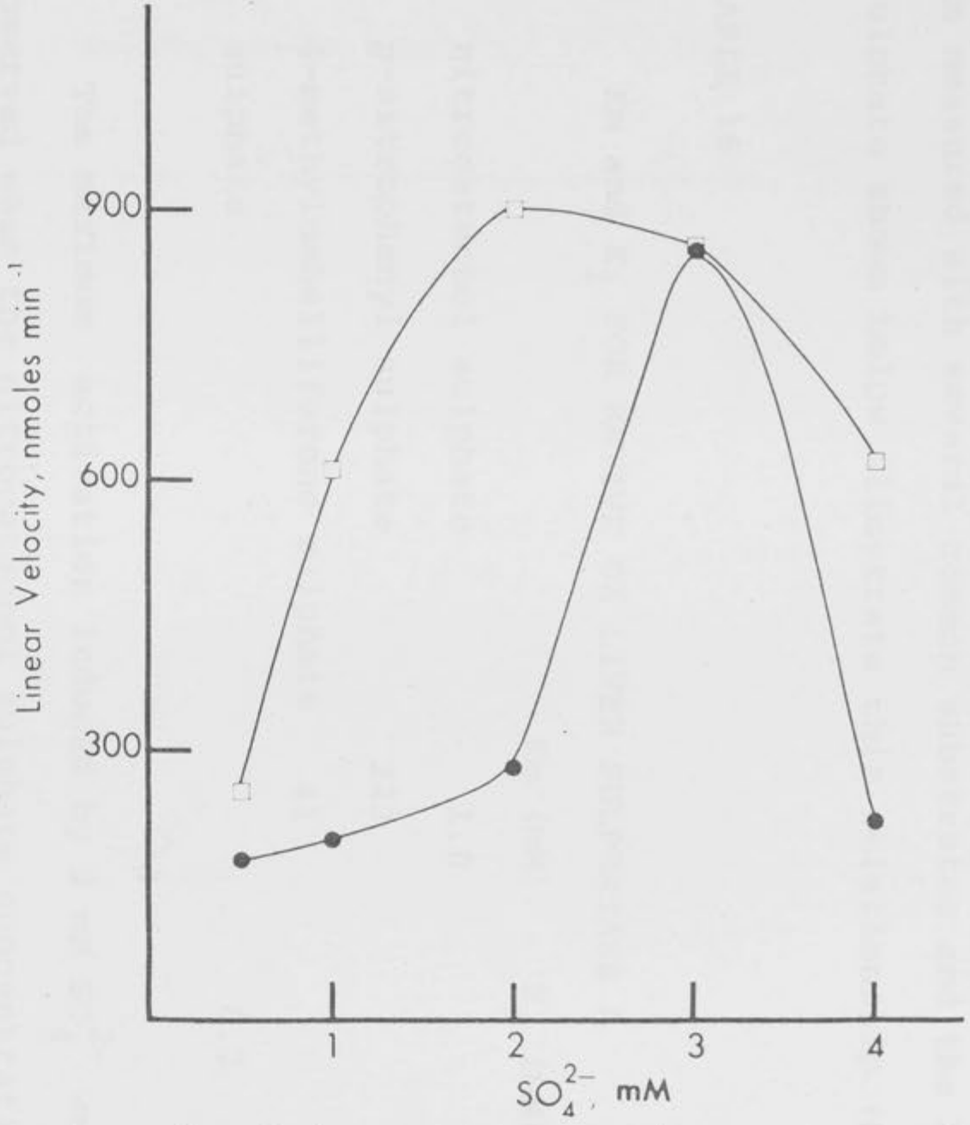


Figure 15 The data of Figure 14 plotted as a function of sulphate concentration. The various nitrocatechol sulphate concentrations are: 0.5 (○), 1.0 (■), 2.0 (□), 3.0 (△), and 4.0 (●) mM.

constants for this substrate and sulphate. The values for Km measured with several common substrates and the Ki of sulphate shown below illustrate this relationship (Roy, 1978).

TABLE 16

Km and K_i FOR NATIVE OX LIVER SULPHATASE A

	Km (mM)	K_i (mM)
nitrocatechol sulphate	1.0	
p-nitrophenyl sulphate	223	
4-methylumbelliferone sulphate	41	
sulphate		0.2

The maximum activation induced by 3 mM SO_4^{2-} was observed when the nitrocatechol sulphate concentration was approximately 3 mM but there was not an appreciable difference in the rate obtained between 2 and 4 mM. For comparison, the optimum conditions for activation using the other substrates were 50 mM p-nitrophenyl sulphate and 6 mM SO_4^{2-}, and 10 mM 4-methylumbelliferone sulphate and 1 mM SO_4^{2-}.

Figure 16, a plot of the rate of hydrolysis observed with p-nitrophenyl sulphate 15 minutes after the addition of sulphate versus the concentration of sulphate added, has the same shape as that observed with nitrocatechol sulphate. Both show an initial increase in the observed velocity with increased sulphate concentration followed by a decrease as sulphate inhibition becomes evident. The enzyme's response to the sulphate is thus similar when either

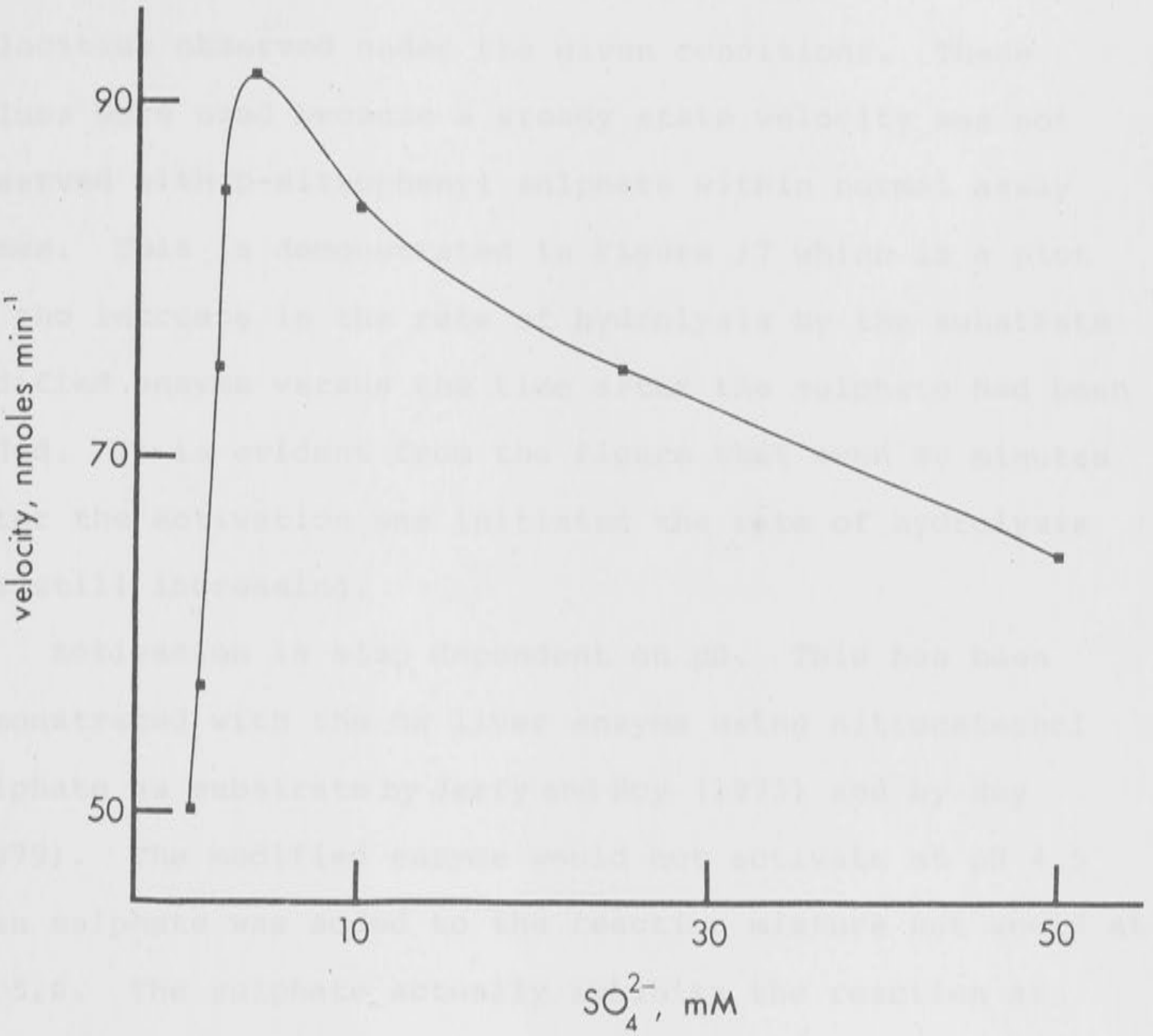


Figure 16 Activation of the substrate-modified enzyme using 50 mM p-nitrophenyl sulphate, pH 6.1, as substrate. The velocity plotted is that observed 15 minutes after the addition of SO_4^{2-}.

nitrocatechol sulphate or p-nitrophenyl sulphate are used as substrate. The velocities plotted in Figure 16 were determined by drawing a tangent to the curve of the pH-stat recording at 15 minutes and do not represent the maximum velocities observed under the given conditions. These values were used because a steady state velocity was not observed with p-nitrophenyl sulphate within normal assay times. This is demonstrated in Figure 17 which is a plot of the increase in the rate of hydrolysis by the substrate-modified enzyme versus the time after the sulphate had been added. It is evident from the figure that even 60 minutes after the activation was initiated the rate of hydrolysis was still increasing.

Activation is also dependent on pH. This has been demonstrated with the ox liver enzyme using nitrocatechol sulphate as substrate by Jerfy and Roy (1973) and by Roy (1979). The modified enzyme would not activate at pH 4.5 when sulphate was added to the reaction mixture but would at pH 5.6. The sulphate actually inhibits the reaction at the lower pH but this would represent inhibition of the activity from residual native enzyme in the preparation which would be expected under these conditions.

Similarly it has been found that with p-nitrophenyl sulphate as substrate the modified enzyme is activated at pH 6.1 but not at pH 5.6 and with 4-methylumbelliferone sulphate as substrate it is activated at pH 5.6 but not at pH 5.2.

B. Ionic Strength

The activity of many enzymes is effected by the ionic

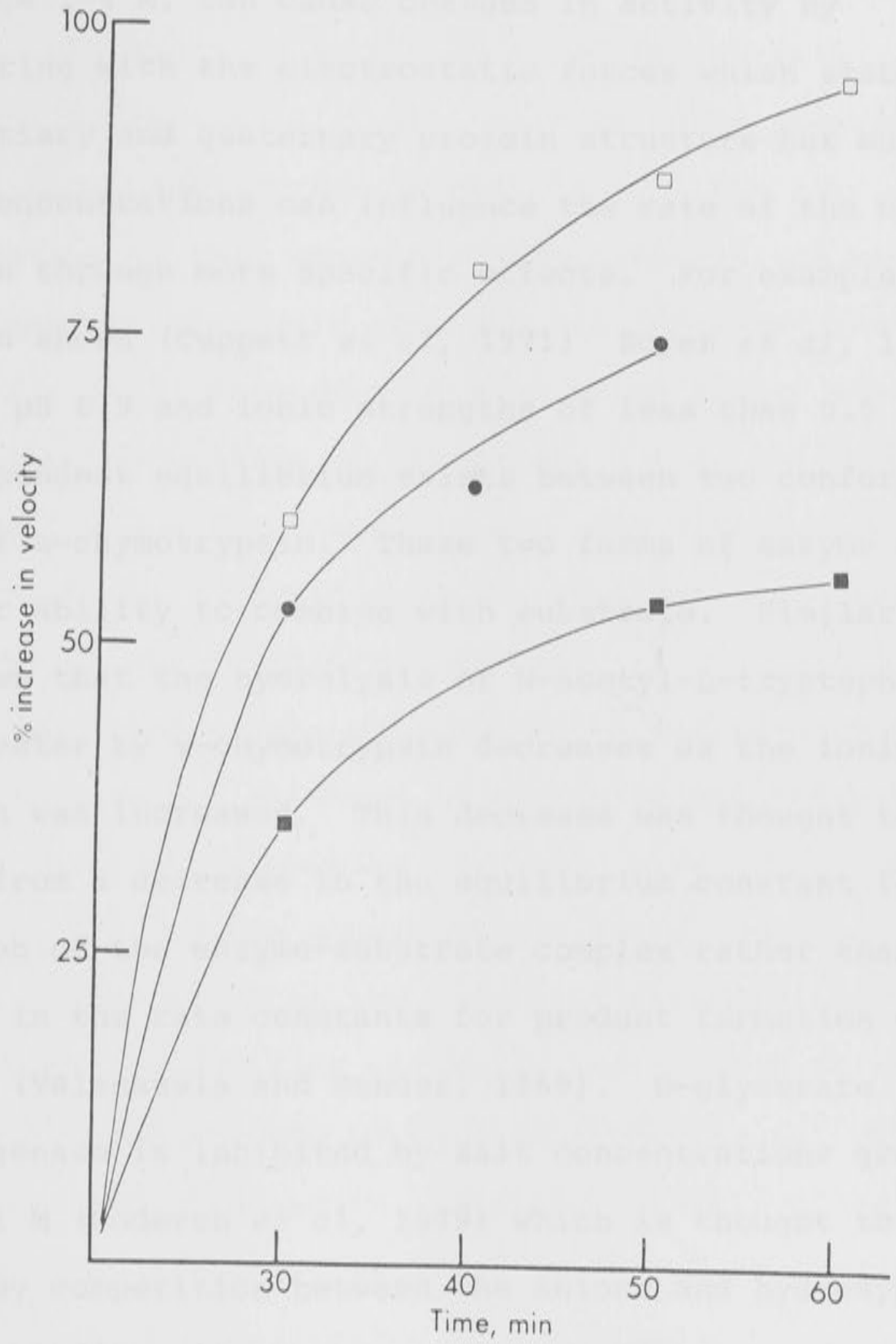


Figure 17 The activation of substrate-modified sulphatase A by SO_4^{2-} with 50 mM p-nitrophenyl sulphate, pH 6.1. The rate of hydrolysis after the addition of 1.5 (■), 3.0 (●), and 6.0 (□) mM SO_4^{2-} is expressed as the increase in the rate over that observed before the addition of SO_4^{2-}.

strength of the assay mixture. High salt concentrations, in the range 1-4 M, can cause changes in activity by interfering with the electrostatic forces which stabilize the tertiary and quaternary protein structure but much lower concentrations can influence the rate of the catalytic reaction through more specific effects. For example it has been shown (Cuppett *et al*, 1971; Royer *et al*, 1971) that at pH 6.9 and ionic strengths of less than 0.5 M a salt dependent equilibrium exists between two conformational forms of α-chymotrypsin. These two forms of enzyme differ in their ability to combine with substrate. Similarly it was shown that the hydrolysis of N-acetyl-L-tryptophan methyl ester by γ-chymotrypsin decreases as the ionic strength was increased. This decrease was thought to result from a decrease in the equilibrium constant for the formation of the enzyme-substrate complex rather than from changes in the rate constants for product formation or release (Valenzuela and Bender, 1969). D-glycerate dehydrogenase is inhibited by salt concentrations greater than 0.1 M (Coderch *et al*, 1979) which is thought to be caused by competition between the anions and hydroxypyruvate for the binding site.

Ionic strength has been shown to influence the activity of sulphatases in different ways. Maengwyn-Davies and Friedenwald (1954) found sodium chloride to be a competitive inhibitor of a preparation of rabbit liver arylsulphatase which had a Ki of approximately 0.5 M. Webb and Morrow (1959) also found chloride to inhibit the hydrolysis of nitrocatechol sulphate by ox liver

sulphatase B. When p-nitrophenyl sulphate was substrate, however, the reaction was activated. The native form of ox liver sulphatase A is activated by different salts (e.g. KCl, $MgCl_2$, $CaCl_2$, $MnCl_2$) when nitrocatechol sulphate is used as substrate (Jerfy and Roy, 1973). The rate of inactivation of this enzyme is also affected and was shown to decrease as the ionic strength was increased (Roy, 1978). Stinshoff (1972) demonstrated with human kidney sulphatase A that the concentration of monovalent anions influenced the velocity found after reactivation. The effect of ionic strength on the activation of the substrate-modified ox liver enzyme was therefore measured.

The modified enzyme was assayed in the pH-stat using 3 mM nitrocatechol sulphate as substrate and activating with 3 mM sulphate while varying the ionic strength. The latter was controlled by the concentration of KCl. The bottom line in Figure 18 shows how the ionic strength affected the activity of the modified enzyme in the absence of any added activator and the upper line shows its influence on the activation by sulphate. Maximum activation was observed when the ionic strength was 0.5. At concentrations greater than this activation is inhibited.

In order to eliminate the possibility that this was a specific effect of either the substrate or the KCl several spectrophotometric assays were done with p-nitrophenyl sulphate as substrate and with sodium acetate controlling the ionic strength. Spectrophotometric assays of substrate-modified sulphatase A using 50 mM p-nitrophenyl sulphate in 0.2 M sodium acetate with activation induced by the addition

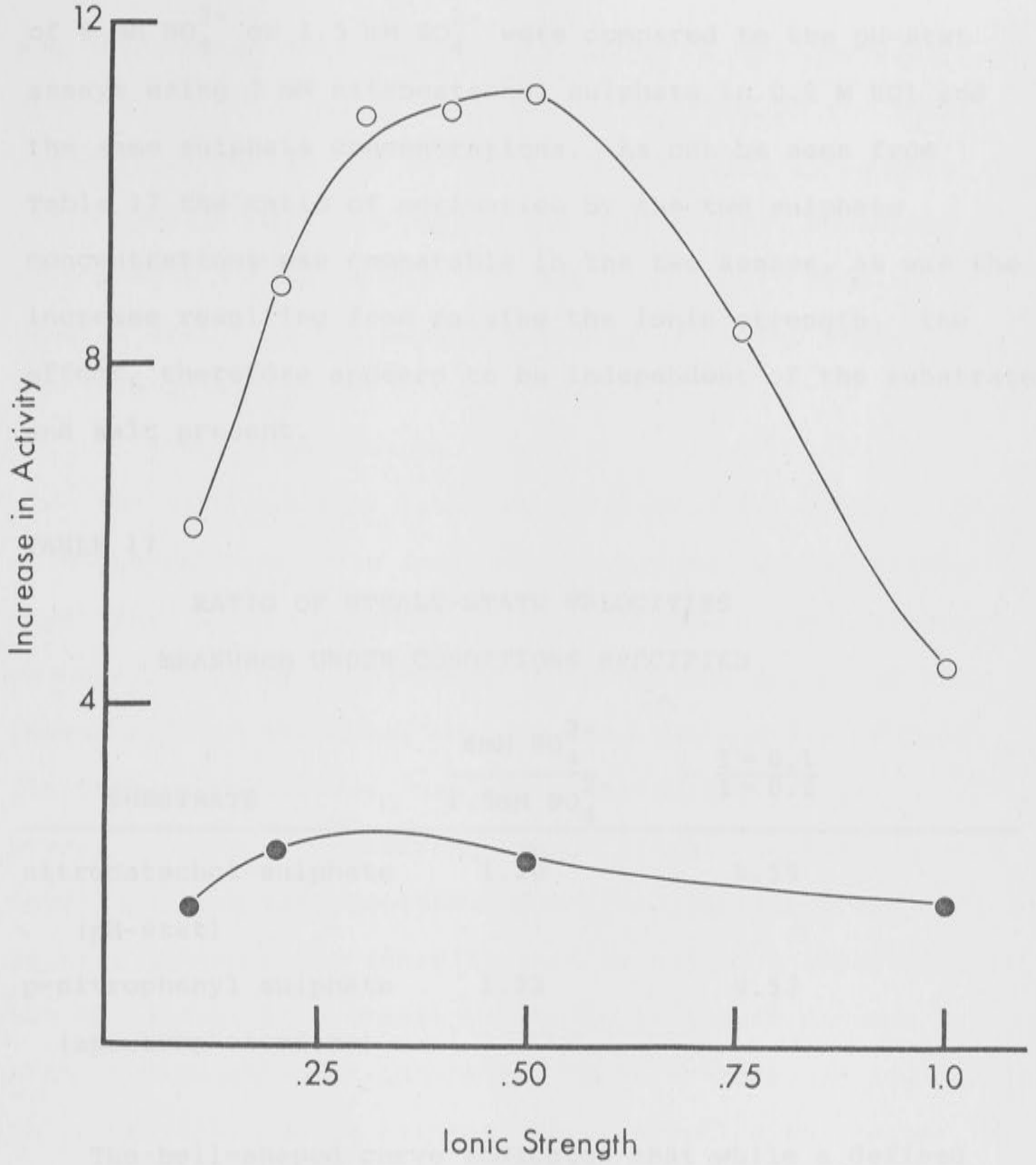


Figure 18 The effect of ionic strength on the activation of substrate-modified sulphatase A. The hydrolysis of 3 mM nitrocatechol sulphate, pH 5.6 was followed in the pH-stat for 60 minutes. The value plotted is the ratio of the velocity observed between 50 and 60 minutes to that observed at 30 minutes. The lower curve (●) shows the effect of ionic strength alone. The upper curve (○) shows the effect of altering the ionic strength when the enzyme is activated by the addition of 3 mM SO_4^{2-} at 30 minutes.

of 6 mM SO_4^{2-} or 1.5 mM SO_4^{2-} were compared to the pH-stat assays using 3 mM nitrocatechol sulphate in 0.2 M KCl and the same sulphate concentrations. As can be seen from Table 17 the ratio of activation by the two sulphate concentrations was comparable in the two assays, as was the increase resulting from raising the ionic strength. The effect, therefore appears to be independent of the substrate and salt present.

TABLE 17

RATIO OF STEADY-STATE VELOCITIES

MEASURED UNDER CONDITIONS SPECIFIED

SUBSTRATE	$\frac{6mM\ SO_4^{2-}}{1.5mM\ SO_4^{2-}}$	$\frac{I = 0.1}{I = 0.2}$
nitrocatechol sulphate (pH-stat)	1.20	0.55
p-nitrophenyl sulphate (spectrophotometer)	1.23	0.52

The bell-shaped curve indicates that while a defined salt concentration can increase the activity of the modified enzyme the effect is lost if this concentration is exceeded. Such a response could be due to the increase in the dielectric constant of the solvent. This might influence the activity of the activated enzyme by increasing the orientation effects of the solvent molecules around charges or developing charges and thus affect bond-breaking or product release steps by strengthening external bonds

and decreasing the electrostatic energy of the charged species. The following decrease in activity as the ionic strength is raised may be due to effects such as reducing the association of the enzyme and substrate or activator or, affecting a rate constant. It may also result from a general change in the enzyme conformation which reduces its specific activity.

C. Kinetic Parameters

The data obtained from the pH-stat assays using nitrocatechol sulphate and SO_4^{2-} at various concentrations were first analyzed graphically to determine the maximum velocity, apparent equilibrium constant and degree of the reaction. The graphical methods used (direct linear plot, Lineweaver-Burk, Hanes, and Eadie-Scatchard) are all standard methods of plotting enzyme kinetic data which were developed from rate equations involving initial velocities. In this instance the velocity used is not an initial velocity but the linear rate obtained between 20 and 30 minutes after the addition of sulphate. These methods are applicable in a restricted sense however, for the following reasons. Initial velocities were used in the derivation of these equations as they eliminate the need to consider the back reaction and effects which may result from product accumulation or depletion of substrate. With the activation of the modified enzyme there is no evidence for the existence of a back reaction and the experiments were designed such that the concentration of product, and therefore of substrate utilized, is very small compared to the initial substrate concentration and concentration of sulphate added.

The linear velocity shows that the reaction scheme given on page 121 has reached a steady state. The parameters calculated in this way are purely empirical values and cannot be defined in terms of a specific mechanism.

Although treatment of the data in this way disregards the initial stage of activation when the observed velocity is not linear with time and gives no indication of the complexity of the constants calculated, some general information can be obtained. As seen in Table 18 a maximum velocity of 67 μmoles min^{-1} mg^{-1} and a Km of 2 mM was calculated from the graphs mentioned above. All lines were fitted visually, eliminating those points beyond the concentration where substrate inhibition became evident. An example of a Lineweaver-Burk plot is shown in Figure 19 to demonstrate the upward curve of the points at high substrate concentration indicative of substrate inhibition and how the line was placed. There was little variation in the values obtained using these four plots and few points were obviously off the lines drawn.

TABLE 18

KINETIC CONSTANTS FOR THE ACTIVATED SUBSTRATE-MODIFIED SULPHATASE A

Method of determination	Km (mM)	V (μmoles min^{-1} mg^{-1})
Direct Linear Plot	2.0	66
Lineweaver-Burk Plot	2.1	69
Hanes Plot	2.0	66
Eadie-Scatchard Plot	2.0	67

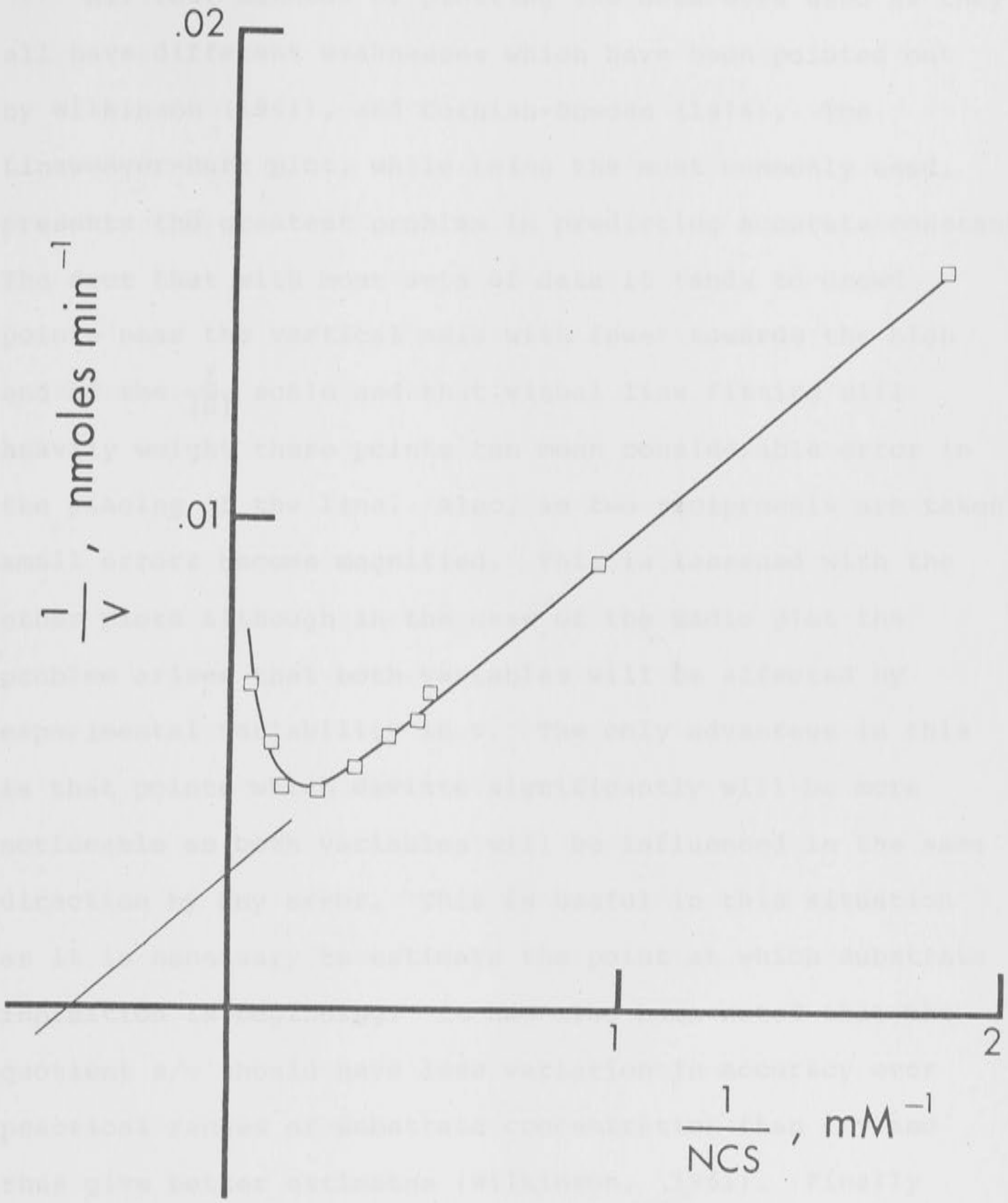


Figure 19 Lineweaver-Burk plot of data obtained by assaying the substrate-modified enzyme in 3 mM nitrocatechol sulphate using 3 mM sulphate as activator. As described in the text, the linear velocity was taken as v. The upward curve of the plot at high substrate concentrations is indicative of substrate inhibition.

All four methods of plotting the data were used as they all have different weaknesses which have been pointed out by Wilkinson (1961), and Cornish-Bowden (1976). The Lineweaver-Burk plot, while being the most commonly used, presents the greatest problem in predicting accurate constants. The fact that with most sets of data it tends to crowd points near the vertical axis with fewer towards the high end of the $\frac{1}{[S]}$ scale and that visual line fitting will heavily weight these points can mean considerable error in the placing of the line. Also, as two reciprocals are taken, small errors become magnified. This is lessened with the other plots although in the case of the Eadie plot the problem arises that both variables will be affected by experimental variability in v. The only advantage in this is that points which deviate significantly will be more noticeable as both variables will be influenced in the same direction by any error. This is useful in this situation as it is necessary to estimate the point at which substrate inhibition is beginning. It has also been noted that the quotient s/v should have less variation in accuracy over practical ranges of substrate concentration than 1/v and thus give better estimates (Wilkinson, 1961). Finally the direct linear plot can be shown to result in less statistical error as the data is plotted directly. This also makes obvious points which do not conform to the rest of the data (Cornish-Bowden and Eisenthal, 1978).

Although the values obtained by these different plots were very similar the data was also analyzed statistically to increase the accuracy of the estimates by applying

appropriate weighting factors to the points and also to obtain an estimate of the precision of the data. It was necessary, however, to first plot the data and visually fit a line before using the statistical method in order to determine the point at which inhibition and thus irregularities in the curve began. Points beyond this limit were deleted and the remaining data was used to compute V and K_m by the method of Wilkinson (1961). The values obtained in this way were 56 $\pm$ 4 µmoles min^{-1} mg^{-1} for V and 1.5 $\pm$ 0.3 for K_m. The line fitted by this method is shown with the one fitted by eye in Figure 20 for comparison.

The variation caused by altering the concentration of sulphate added as activator to an otherwise similar set of assays is shown in Table 19. Values of V and K_m were calculated using the Wilkinson method, as before, from a set of assays where the sulphate concentration was kept constant and the substrate concentration varied from 0.1 mM to the concentration where inhibition began. These values do not vary linearly with activator concentration suggesting that a complex relationship exists between the concentration of substrate and activator and the observed velocity.

The fact that the four plots given in Table 18 are linear in this restricted concentration range where substrate inhibition is not obvious indicates that the reaction is first order with respect to substrate once it has reached equilibrium. It is unfortunate that linear rates were not obtained with p-nitrophenyl sulphate and 4-methylumbelliferone sulphate within a reasonable length

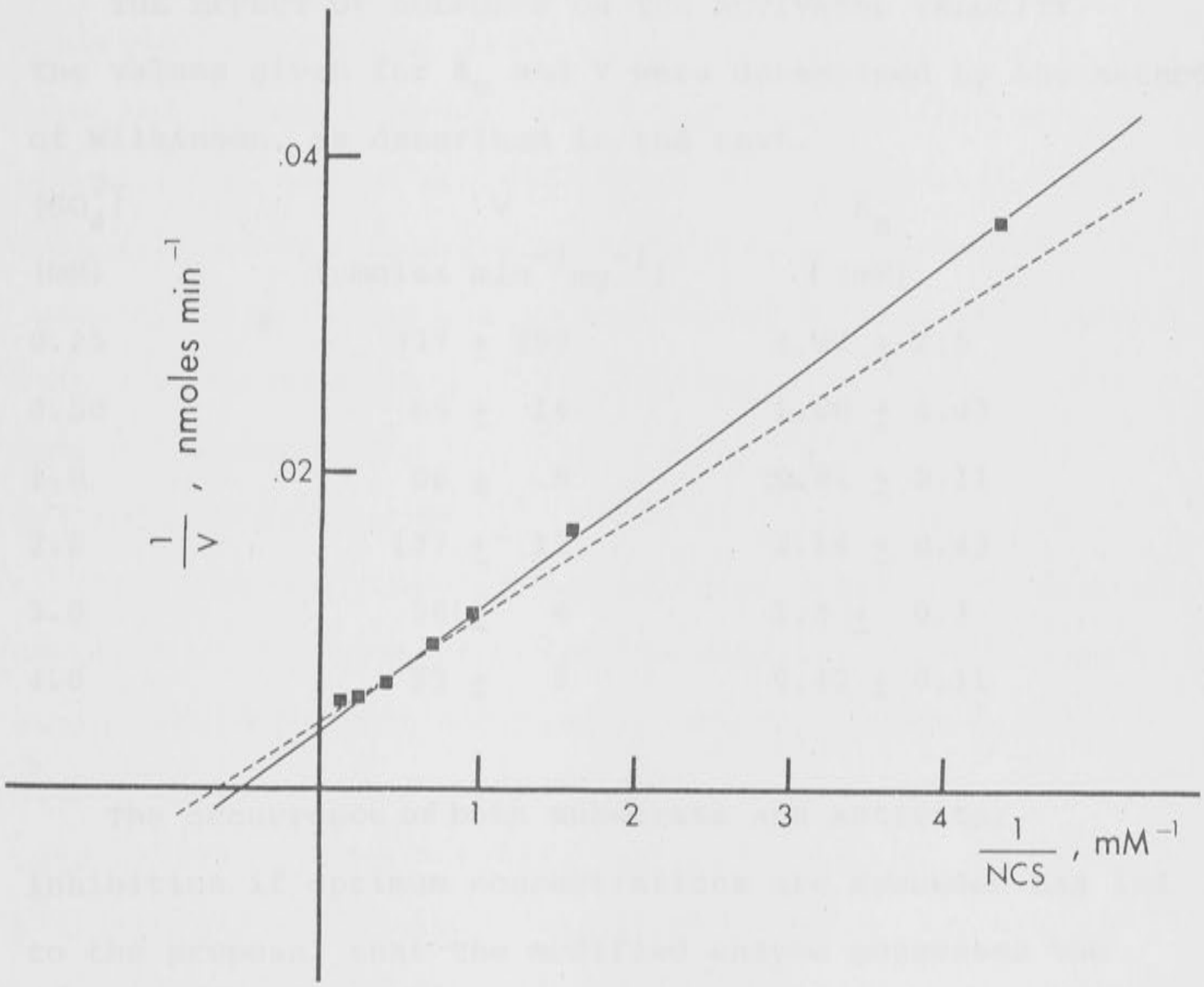


Figure 20 Determination of V and Km by visual fitting of a line through points on a double-reciprocal plot (——) and by the method of Wilkinson (----). In the latter case the values have been plotted after calculation.

of time and the relationship could not be verified with another substrate.

TABLE 19

THE EFFECT OF SULPHATE ON THE ACTIVATED VELOCITY

The values given for K_m and V were determined by the method of Wilkinson, as described in the text.

$[SO_4^{2-}]$ (mM)	V (μmoles $min^{-1}mg^{-1}$)	K_m (mM)
0.25	337 ± 399	4.87 ± 2.5
0.50	65 ± 16	1.00 ± 0.43
1.0	66 ± 9	0.61 ± 0.11
2.0	127 ± 17	2.14 ± 0.43
3.0	56 ± 4	1.5 ± 0.3
4.0	33 ± 3	0.43 ± 0.11

The occurrence of both substrate and activator inhibition if optimum concentrations are exceeded has led to the proposal that the modified enzyme possesses two binding sites although this has never been proven. Because of the substrate inhibition and the fact that two different ligands can be bound, use cannot be made of the Hill Plot to determine the number of binding sites.

The number of sites capable of binding sulphate could theoretically be determined by equilibrium dialysis against $^{35}SO_4^{2-}$. A known quantity of the substrate-modified enzyme was dialyzed against 10 mM $^{35}SO_4^{2-}$ in 0.01 M Tris-HCl pH 7.4, 0.15 M NaCl for 2 days and aliquots of the enzyme solution and the buffer counted. The results indicated

that 36 moles of SO_4^{2-} had bound to 1 mole of sulphatase A which presumably reflects extensive non-specific binding.

It is also possible to determine the number of binding sites by measuring the rate of dialysis of radioactively labelled ligand from a solution containing the enzyme (Colowick and Womack, 1969). The rate at which the labelled ligand dialyzes out of the solution will be proportional to the concentration of unbound ligand. This method, however, requires that the ligand equilibrate immediately with the enzyme when added to the solution and it cannot be assumed that this is the case in the activation reaction.

5.4 RATE OF ACTIVATION

The progress curve of the reaction between the time of addition of the activator and the attaining of the steady state should provide additional information about the mechanism involved. Theoretically, the use of integrated rate equations does not have the limitation of requiring all intermediate steps in the reaction to be treated as one. Therefore, if kinetic data can be fitted to suitable rate equations, postulated models can be confirmed and rate constants for individual steps in the mechanism determined. For simple mechanisms this has indeed been found to be the case. The problem still remains, however, that when complex mechanisms are involved it is difficult, if not impossible to distinguish between different mechanisms. Even when data are calculated from the derived equations differences are often undetectable and it may be found that several rate equations with different kinetic

constants can be fitted to a given set of experimental data. Such mechanisms are commonly simplified so that the equations to be fitted do not require more accuracy in the data than it is possible to attain but if this is done it must be remembered that the empirical rate constants may in reality encompass several individual constants.

5.41 Preincubation with Sulphate

The lag phase could not be eliminated by preincubating the enzyme with the activator. Incubating the modified enzyme in 3 mM SO_4^{2-}, pH 5.6, 0.1 M KCl for 10 minutes in the pH-stat did not alter the time course of the activation when the solution was made 3 mM nitrocatechol sulphate by the addition of 0.6 ml of a 50 mM nitrocatechol sulphate solution pH 5.6. This indicates either that the initial binding of activator is not the rate limiting step of the activation mechanism or that the sulphate is only able to bind after the substrate. The possibility also exists that FI_2 has been formed during the incubation with sulphate and that a slow formation of FI from FI_2 is causing the lag time. Similarly in the normal activation assay where the enzyme is incubated with substrate for 30 minutes before the addition of sulphate a complex FS_2 may be formed and a slow release of S to give FS may be the rate limiting step in the activation in this case. Evidence that this is unlikely to be occurring was obtained by assaying the modified enzyme with nitrocatechol sulphate and sulphate without prior exposure to either. A reaction mixture of 3 mM nitrocatechol sulphate, pH 5.6, 0.1 M KCl, 3 mM K_2SO_4 was equilibrated as usual in the pH-stat and

substrate-modified enzyme was added at zero time. A preparation of the modified enzyme which had been inactivated twice (see section 4.14) and therefore contained only 3% native enzyme was used to reduce the problem of having two forms of sulphatase A present at the same time. The progress curve produced clearly showed a slow rate of activation with a lag time comparable to that found in the normal assay. This indicates that the above suggestion is unlikely and that it is either an initial binding step or a step occurring after the binding of both S and I which is rate limiting rather than the release of a bound ligand. It seems improbable that the rate limiting step is different under the various assay conditions because the lag times observed in each case are identical.

5.42 Other Activators

Before investigating the slow rate of activation observed when sulphate is added to an assay mixture containing substrate-modified sulphatase A, the course of activation induced by other substances was followed to see if similar time lags were evident. Other anions, such as fluoride, phosphate and pyrophosphate, had been shown to activate the modified enzyme but this had been deduced from assays of the native enzyme where these compounds were present from the beginning of the reaction. Their activation of the isolated substrate-modified enzyme had not been studied. The modified enzyme was therefore activated in the pH-stat with several different anions. The shapes of the kinetic curves produced in each case were similar. With pyrophosphate, sulphite, selenate and selenite, as well as with sulphate, a lag time was evident

before the maximum velocity was reached. Table 20 gives the concentrations of nitrocatechol sulphate and activator used and indicates the length of time which elapsed in each case before a linear velocity was observed. The ratio of the final velocity, taken between 20 and 30 minutes after the addition of activator, to the initial velocity, taken between 2 and 3 minutes after the addition of the enzyme, is also given for comparison. The mechanism of activation appears to involve the same slow transition with all of these activators. Because SO_3^- is readily oxidized the concentration given may be in error. It should be noted however, that 0.2 mM SO_4^{2-} would not produce the observed activation and therefore that the measured response is not due to the SO_3^- becoming fully oxidized in the reaction mixture.

In contrast to these results obtained with sulphite, Waheed and Van Etten (1980b) found that the rabbit liver modified sulphatase A was not activated by this anion, nor was the residual activity in the preparation inhibited. The cause of this difference is not known.

TABLE 20

ACTIVATION OF SUBSTRATE-MODIFIED SULPHATASE A BY VARIOUS ANIONS

Substrate	Activator	Time to Reach Equilibrium	*Ratio of Final to Initial Velocity
3 mM NCS	3 mM SO_4^{2-}	6 min	3.57
5 mM NCS	.25 mM $P_2O_7^{2-}$	16	3.85
3 mM NCS	.2 mM SO_3^-	5	4.55
3 mM NCS	10 mM SeO_4^{2-}	26	2.44
3 mM NCS	10 mM SeO_3^-	19	1.56

* The initial velocity was taken as the slope between 2 and 3 minutes after the reaction was initiated and the final velocity as the linear rate usually observed between 20 and 30 minutes after the addition of the activator. With SeO_4^{2-} the final velocity was taken between 28 and 38 minutes.

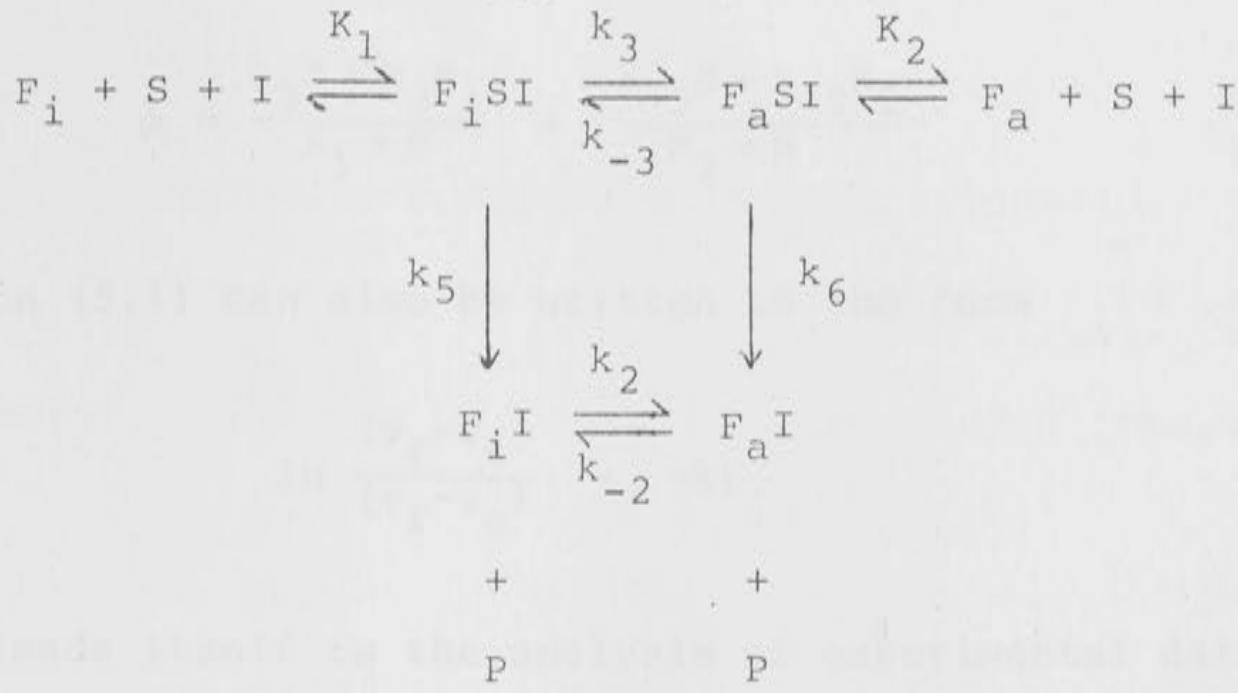
5.5 RATE CONSTANTS FOR ACTIVATION

Three different approaches were followed in analyzing the activation stage of the reaction. The methods used vary in their complexity and consequently in the amount of information which they can supply. The data was first analyzed simply with regard to the time between the addition of sulphate and a linear velocity being observed. It was then attempted to define a mechanism and determine more individual rate constants.

5.51 Time Required for Activation

If a series of assays are done over a wide range of substrate and activator concentrations it becomes evident that the time required for the rate of hydrolysis to become linear is dependent on these concentrations. In order to estimate the rate at which the steady state is reached without considering the mechanism of activation or the concentrations of substrate or activator present the data were fitted to the general equation given below. This equation (Frieden, 1970) has been shown to describe many reactions where a change in the rate of an enzyme-catalyzed reaction is observed to take place over a measurable length of time. It makes no attempt to define the mechanism responsible and gives an overall rate constant for a given change in the observed velocity. Any dependence of this rate on the concentration of substrate or effectors will be contained in this rate constant. In applying this equation it is assumed that the overall reaction is irreversible, the substrate concentration remains constant and there is no product inhibition. These criteria are met by the activation reaction under the experimental conditions employed.

The reaction scheme written in terms of the activation of the modified enzyme is



It is assumed that $F_i + S + I \rightleftharpoons F_iSI$ and $F_a + S + I \rightleftharpoons F_aSI$ are in rapid equilibrium and therefore that the initial binding step is not rate limiting. If k_3, k_{-3}, k_2 and k_{-2} are small enough then the change in velocity will occur over a measurable length of time. The time dependence of the velocity in this mechanism is given by Frieden as

$$v_t = v_f + (v_o - v_f)e^{-kt} \qquad (5.1)$$

where

v_f = final velocity

v_o = velocity at time zero

v_t = velocity at time t

k = a complex rate constant for the change in velocity

This equation is independent of the relative values of k_5 and k_6 and is not affected by the fact that k_5 is apparently very small compared to k_6 in this case. K_2 in the above mechanism must also be very small to be consistent with the postulated scheme (page 121). The rate constant k will be dependent on S, A, K_1, K_2 and the other rate constants and, under the conditions specified

$$k = \frac{k_3 S + k_2 K_1}{K_1 + S} + \frac{k_{-3} S + k_{-2} K_2}{K_2 + S} \qquad (5.2)$$

Equation (5.1) can also be written in the form

$$\ln \frac{(v_f - v_t)}{(v_f - v_o)} = -kt \qquad (5.3)$$

which lends itself to the analysis of experimental data. A plot of $\ln \frac{(v_f - v_t)}{(v_f - v_o)}$ versus time will give a straight line with slope equal to -k. In this case v_f was taken as the steady state velocity, v_t were measured, by drawing tangents to the progress curve, at one minute intervals from the time of addition of sulphate for 10 minutes and v_o was taken as being zero.

Table 21 gives the values for k obtained in this way. Not all of the assays could be analyzed due to irregularities in the titration curve which occurred in some cases when the sulphate was added or because of the very shallow slope produced at early times by the reaction. Several of these rate constants were used to calculate, by means of the integrated form of the above equation (5.4), the concentration of product produced during the first 10 minutes of the reaction. These data are given in Table 21.

$$P = v_f t - \frac{v_f - v_o}{k} (1 - e^{-kt}) \qquad (5.4)$$

Good agreement is observed between a number of the observed and calculated concentrations although measured values are generally higher than the calculated values, in some cases quite considerably. This may be due to the error

TABLE 21

RATE CONSTANTS FOR SULPHATE-INDUCED ACTIVATION

SO_4^{2-} (mM)	NCS (mM)	k	Product Concentration at 10 minutes in %	
			Calculated	Measured
0.25	0.2	.08		
	0.4	.03	45	77
0.50	0.2	.11	48	95
	0.5	.02		
1.0	0.1	.17	11	15
	0.2	.15	21	24
	0.3	.07	11	20
	0.4	.11	78	96
	1.0	.02	31	76
	2.0	.02	21	52
	5.0	.05	73	69
2.0	0.1	.20	64	61
	0.15	.13	77	79
	0.2	.06	46	43
	0.3	.02	24	56
	1.0	.03	39	44
3.0	0.25	.12		
	0.5	.11		
	0.75	.18	60	73
4.0	0.1	.09		
	0.15	.08	18	28
	0.2	.08	23	35
	0.25	.06		
	0.3	.05	31	35
	1.0	.02		

involved in measuring velocities during initial times when the slope of the pH-stat recording is very shallow. There is appreciable scatter in these values of k and this, along with the limited amount of data, meant that it was not possible to find a mathematical correlation between the concentrations of substrate and sulphate present and the rate constant. While there is no apparent difference between the values at different sulphate concentrations there is a general decrease in the rate constant as the concentration of nitrocatechol sulphate is increased and the sulphate concentration is held constant.

5.52 Two Step Mechanism

In order to further define the relationship between the rate at which a linear velocity is attained and the magnitude of that velocity two rate constants were defined within the overall reaction. This is a gross simplification of the kinetic mechanism and the rate constants defined will represent the overall rate produced by several intermediate steps.

If the reaction mechanism is written as before, an equation for the rate of product formation can be derived as follows, assuming the reaction to be irreversible.

$$F_i + S + I \underset{}{\overset{k_1}{\rightleftharpoons}} FaSI \xrightarrow{k_2} F_i + P + I$$

The observed rate of product formation can be written as

$$\frac{dP}{dt} = k_2[F_aSI] \qquad (5.5)$$

The rate of increase of product formation before a steady state is reached is therefore

$$\frac{d^2P}{dt^2} = k_2 \frac{d[F_aSI]}{dt} \qquad (5.6)$$

As

$$\frac{d[F_aSI]}{dt} = k_1[S][I][F_i] - (k_{-1} + k_2)[F_aSI]$$

$$= k_1[S][I][F_t - F_aSI] - (k_{-1} + k_2)[F_aSI]$$

$$= [k_1[S][I][F_t]] - [k_1[S][I] + k_{-1} + k_2][F_aSI]$$

$$\frac{d^2P}{dt^2} = k_1k_2[S][I][F_t] - (k_1[S][I] + k_{-1} + k_2)\frac{dP}{dt} \qquad (5.7)$$

If $\frac{[S][I]}{[F_t]}$ is assumed to be large so that the substrate concentration does not change during the assay then this equation can be solved (Roughton, 1954) and the concentration of product at any given time may be written as

$$P = P_o + \frac{k_2k_1[S][I][F_t]t}{k_1[S][I] + k_{-1} + k_{-2}} + \frac{k_2k_1[S][I][F_t]}{(k_1[S][I] + k_{-1} + k_2)^2} \cdot$$

$$\left[e^{-(k_1[S][I] + k_{-1} + k_2t)} - 1\right] \qquad (5.8)$$

It can be seen from this equation that as time increases the exponential term will become negligible and the system will pass into a steady state, that is, P will accumulate linearly with time. Before this linear rate is reached product will accumulate exponentially. By expanding the exponential term ($e^x = 1 + x + \frac{x^2}{2!} + \frac{x^3}{3!}$ ....) and substituting the first three terms into (5.8) the concentration of

product is defined as

$$P = \frac{k_2 k_1 [S][I][F_t] t^2}{2} \qquad (5.9)$$

A plot of P versus t^2 should therefore give a straight line with slope of $\frac{k_1 k_2 [S][I][F_t]}{2}$.

If the activation data obtained here is graphed in this way a curve with constantly decreasing slope is produced. The initial points do form a straight line, but the slope remains constant only for approximately half a minute, i.e. 10 points taken in the first 25 seconds after the addition of sulphate fall on a straight line. The nature of these assays is such that the titration has stopped before the sulphate is added and begins again as activation is initiated. Because of the error inherent in the initial titration by the pH-stat, as discussed in Chapter 2, little weight can be placed on readings taken in the first 30 seconds. Even if this were not the case, the slope obtained over this short time span cannot be relied on as a measurement of the rate at which the modified enzyme is activated as it represents less than 5% of the time required for the hydrolysis rate to become constant. Other factors and equilibria must therefore be considered in order to estimate the rate changes which are observed.

The relationship between the rate at which a linear velocity is reached and the concentrations of substrate and activator can be estimated by another approach, however.

Once a steady state has been reached equation (5.8) can be written as

$$P = \frac{k_2 k_1 [S][I][F_t]t}{k_1[S][I] + k_{-1} + k_2} - \frac{k_2 k_1 [S][I][F_t]}{(k_1[S][I] + k_{-1} + k_2)^2} \qquad (5.10)$$

From this equation it can be seen that extrapolation of the linear region of the kinetic curve back to the point where the concentration of product is zero, a time will be defined at the intercept of the t-axis where

$$\frac{k_2 k_1 [S][I][F_t]t}{k_1[S][I] + k_{-1} + k_2} - \frac{k_2 k_1 [S][I][F_t]}{(k_1[S][I] + k_{-1} + k_2)^2} = 0 \qquad (5.11)$$

This time is called t_{lag} and reduces to

$$t_{lag} = \frac{1}{k_1[S][I] + k_{-1} + k_2} \qquad (5.12)$$

Figure 21 is an example of how t_{lag} was calculated from the data given by a pH-stat recording. Points were read off the original recording at 1 minute intervals and the product concentration converted to µmoles produced per mg enzyme. These values were replotted for each assay and t_{lag} was obtained from the intercept on the graph. Figure 22 shows the variation of t_{lag} with nitrocatechol sulphate concentration at different concentrations of sulphate.
It is interesting to compare Figure 22 with Figure 14 which shows the steady state velocities reached in each of these assays. A bell shaped curve is produced in each case and the maximum of each curve is in approximately the same

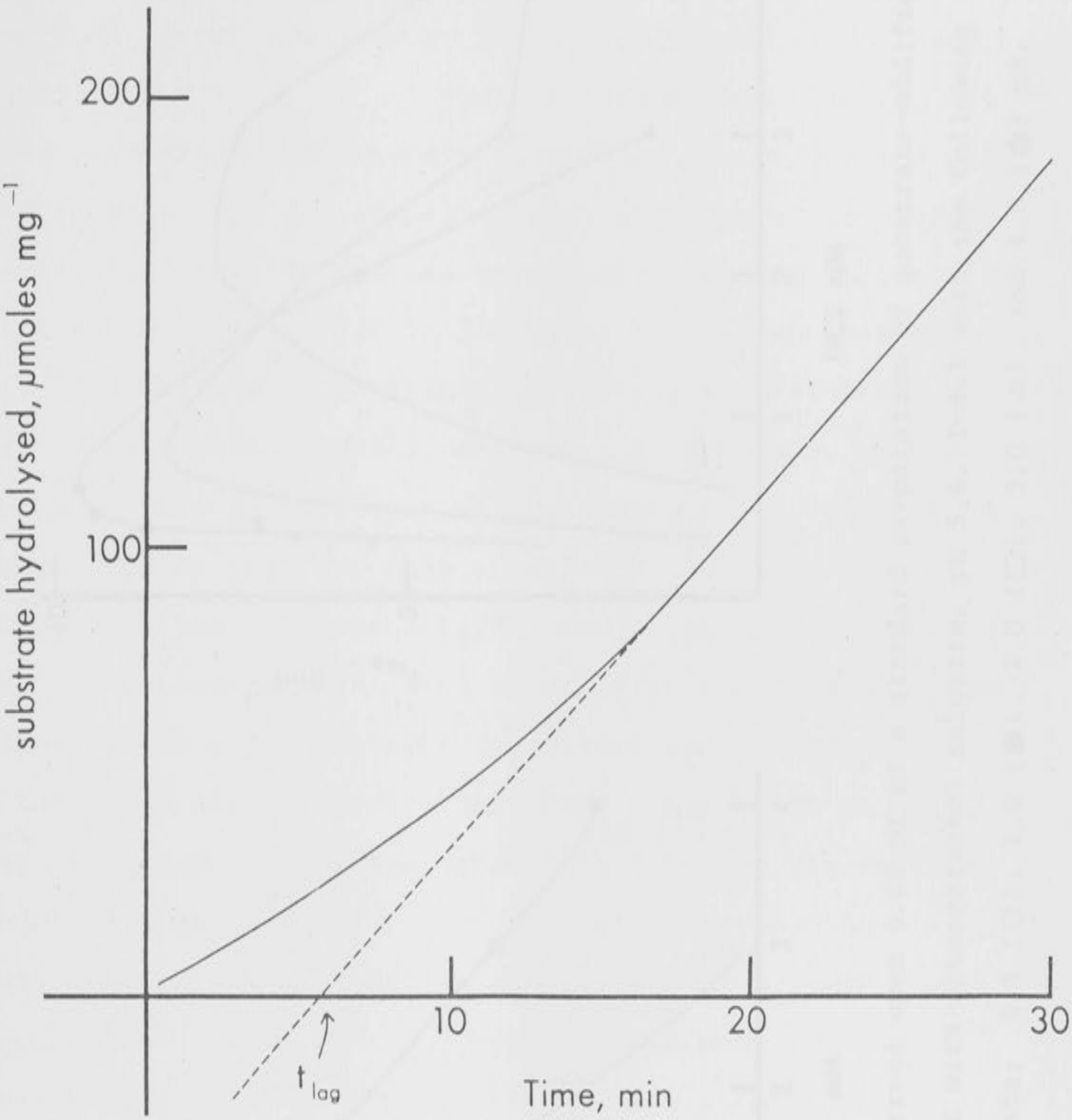


Figure 21 Calculation of t_{lag} from a pH-stat recording. The solid line shows the recording obtained from a pH-stat activation assay after the addition of sulphate converted from % titrated to μmoles product produced per mg enzyme. The dashed line shows how the linear portion of this curve was extended back until it crossed the time axis. This intercept was taken as t_{lag} as defined in the text.

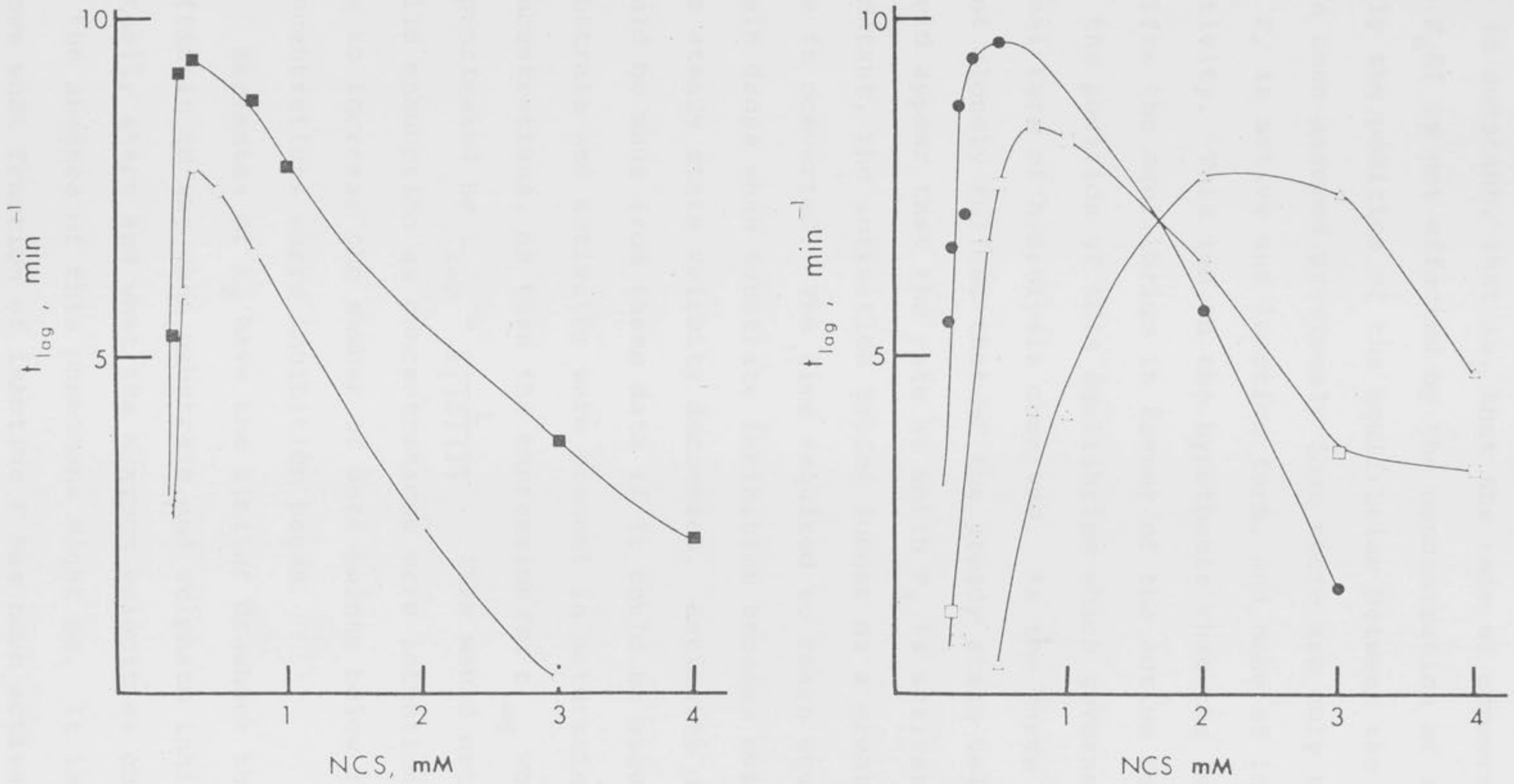


Figure 22 The t_{lag} observed when 0.02 ml of a standard preparation of substrate-modified sulphatase A was assayed with nitrocatechol sulphate, pH 5.6, I=0.1 with the following concentrations of sulphate: 0.5 (○), 1.0 (■), 2.0 (□), 3.0 (△), and 4.0 (●) mM.

position for corresponding curves. This would suggest that k_1 is constant, that is, that the rate of conversion of F_i to F_aSI is not affected by the concentration of sulphate only the position of the equilibrium between the two. It has been assumed previously that there are only two forms of F, an active and inactive form, and none of intermediate activity. This led to the hypothesis that the sulphate shifts the equilibrium in favour of the active form and it is the position of this equilibrium which governs the final rate of hydrolysis observed. As the curve of the t_{lag} plot closely follows that of the steady state velocity it would appear that the rate at which F_i is activated is constant, the activation taking longer as a greater percentage is converted. The time required to reach equilibrium again drops when substrate inhibition becomes evident and the steady state velocity decreases. Estimates of k_1 could be made from these data if it could be assumed that substrate and activator were present in saturating concentrations, as then the expression for t_{lag} would be approximated by $t_{lag} = \frac{1}{k_1[S][I]}$. This would not be a valid assumption as concentrations were intentionally kept low to increase the number of data points below the concentrations where inhibition began.

Estimates of k_2 have the similar drawback that it is difficult to say when substrate and sulphate inhibition actually start and what the maximum velocities observed in the absence of this phenomena might be. It is not known what fraction of inactive F has been activated in each case nor have the inhibition effects been clearly

described. Of the three reactions thought to occur with the substrate-modified enzyme only the one leading to the active form has been considered, i.e.

$$F_i + S + I \rightarrow F_aSI$$
$$F_i + S + S \rightarrow F_iS_2$$
$$F_i + I + I \rightarrow F_iI_2$$

and although it was attempted to minimize the effects of the second two it is not known how well this was accomplished.

5.53 Three Step Mechanism

It is clear from the above that this method is inadequate for quantitative analysis. It is not possible to get numerical values for kinetic constants nor to specify any intermediate steps using this model. An attempt was therefore made to define the mechanism more closely and to fit the data to the rate equations derived from this scheme. The approach taken follows that of Hijazi and Laidler (1973) with modifications made to account for the fact that the modified enzyme (F_i) is thought to have no activity in the absence of an activator. It should be noted that although sulphate is a product of reaction, in this derivation the sulphate concentration added as activator is kept separate from that produced in the course of the reaction. This is done to stress the fact that it is not only sulphate, but also other anions which are not reaction products which have this effect. Keeping the two concentrations separate in this case is mathematically sound as the concentration of sulphate added as activator is

very large compared to that produced by the reaction.

The mechanism may be written schematically as follows

$$\underset{(e_o-m-n)}{F_i} + \underset{(s_o)}{S} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \underset{(m)}{F_iS} + \underset{(a_o)}{I}$$

$$F_iS + I \underset{k_{-a}}{\overset{k_a}{\rightleftharpoons}} \underset{(n)}{F_aSI} \xrightarrow{k_2} F_i + I + \underset{(x)}{X}$$

where F_i = inactive modified enzyme

F_a = active modified enzyme

S = substrate

I = activator

m & n = concentrations of intermediates

x = concentration of products

e_o = initial concentration enzyme

s_o = initial concentration substrate

a_o = initial concentration activator

The differential rate equations for the formation of intermediates and product are

$$\frac{dm}{dt} = k_1 s_o(e_o-m-n) - k_{-1}m - k_a a_o m + k_{-a}n \qquad (5.13)$$

$$\frac{dn}{dt} = k_a a_o m - (k_{-a} + k_2)n \qquad (5.14)$$

$$\frac{dx}{dt} = k_2 n \qquad (5.15)$$

Replacing the differentials by operators gives

$$(P + k_1 s_o + k_{-1} + k_a a_o)m = k_1 s_o e_o - (k_1 s_o - k_{-a})n \qquad (5.16)$$

$$(P + k_{-a} + k_2)n = k_a a_o m \qquad (5.17)$$

$$n = \frac{k_a a_o m}{P + k_{-a} + k_2} \qquad (5.18)$$

Substituting this expression for n into equation (5.16) gives

$$m = \frac{k_1 s_o e_o (P + k_{-a} + k_2)}{(P + k_1 s_o + k_{-1} + k_a a_o)(P + k_{-a} + k_2) + k_a a_o (k_1 s_o + k_{-a})}$$

$$= \frac{k_1 s_o e_o \ (P + k_{-a} + k_2)}{(P + \lambda_1)(P + \lambda_2)} \qquad (5.19)$$

where λ_1 and λ_2 are the roots of the denominator.
Expressions for these roots were found by Laidler to be

$$\lambda_1 = \frac{1}{2} [-R - \sqrt{R^2 - 4Q}]$$

$$\lambda_2 = \frac{1}{2} [-R + \sqrt{R^2 - 4Q}]$$

where $R = k_1 s_o + k_{-1} + k_a a_o + k_{-a} + k_2$

$$Q = k_{-a}(k_1 s_o + k_{-1}) + k_2(k_1 s_o + k_{-1} + k_a a_o) + k_1 s_o k_a a_o.$$

Further substituting equation (5.18) into (5.15) gives

$$x = \frac{k_2 k_a a_o k_1 s_o e_o}{P(P+\lambda_1)(P+\lambda_2)} \qquad (5.20)$$

The expression for the rate of formation of product at time t is thus

$$x = \frac{k_2 k_1 s_o e_o (k_a a_o)}{\lambda_1 \lambda_2} t + \frac{k_2 k_1 s_o e_o (k_a a_o - \lambda_1)}{\lambda_1^2 (\lambda_2 - \lambda_1)} (e^{-\lambda_1 t} - 1)$$

$$+ \frac{k_2 k_1 s_o e_o (k_a a_o - \lambda_2)}{\lambda_2^2 (\lambda_1 - \lambda_2)} (e^{-\lambda_2 t} - 1) \qquad (5.21)$$

which reduces to

$$x = \frac{k_2 s_o e_o (\alpha a_o)}{K_m (1 + \alpha \frac{k_2}{k_1 K_m} a_o) + s_o (1 + \alpha a_o)} t + B_1 (e^{-\lambda_1 t} - 1) + B_2 (e^{-\lambda_2 t} - 1)$$

(5.22)

when values for λ_1 and λ_2 are substituted, K_m set equal to $\frac{k_{-1}}{k_1}$ and α equal to $\frac{k_a}{k_{-a}+k_2}$ and where B_1 and B_2, the exponential coefficients, are equal to those in equation (5.21). This is therefore the velocity measured.

It should be noted at this point that if the mechanism is outlined such that F_aSI decomposes to $F_aI + X$ the velocity equation becomes

$$x = e_o \left(\frac{k_{-a}+k_2}{k_a a_o} + 1\right) - \sum_{i=1}^{3} \frac{k_1 k_2 s_o e_o (k_{-a}+k_2+k_a a_o - \lambda_i)}{\lambda_i (P - \lambda_i)} e^{-\lambda_i t}$$

(5.23)

By comparing equations (5.22) and (5.23) the differences in the progress curves observed as a result of each of these underlying mechanisms can be predicted. If F_aSI breaks down to form $F_aI + X$ and F_aI cannot bind S, that is the

order of binding of substrate and activator is critical, then a steady state will not be reached and the limiting concentration of product will be equal to the first term in equation (5.23). If F_aI is stable and able to bind S then a linear velocity will be established, the magnitude of which will depend on the concentration of F_aI and the rate constant governing product formation and release. If I is released along with X equation (5.22) applies and, as it contains a term linear in t, a linear velocity will be observed with

$$v = \frac{k_2 s_o e_o (\alpha a_o)}{K_m \left(1 + \frac{\alpha k_2}{k_1 K_m} a_o\right) + s_o (1 + \alpha a_o)} \qquad (5.24)$$

once a steady state has been reached. As the active form of the modified enzyme could not be isolated and pre-incubation with I did not alter the time course of this reaction, it is thought that the latter is the better representation of what is occurring.

Theoretically this rate equation can be applied to experimental data to obtain values for the parameters defined. The steady state portion of the curve must first be analyzed as above. A value of V and K_m was calculated for each set of data by the Wilkinson method. Substrate concentrations were specifically kept low (below 1 mM) for this set of assays to avoid the need of eliminating points due to substrate inhibition and 5-9 data points were used in each calculation. From equation (5.22), $\frac{1}{V}$, or the $\frac{1}{v}$ intercept of the data plotted as a double-reciprocal plot,

will equal $\frac{1 + \alpha a_o}{k_2 e_o \alpha a_o}$. If this value is then plotted against the concentration of activator used a curve with slope equal to $-\frac{1}{k_2 e_o \alpha a_o^2}$ is produced. This will give a line which becomes undefined at the ordinate axis (a_o=0) and whose slope becomes vanishingly small as a_o becomes large. When the 1/V values measured are plotted in this way a curve of this shape is not produced.

The small number of points obtained and the large errors associated with these values made it impossible to draw a reliable curve through them. The general trend, however, appears to be toward higher values of 1/V as a_o is increased with no levelling off evident in this concentration range. This suggests that the activation mechanism is not accurately described by this model. The discrepancy may arise if the activated enzyme is stable in the assay mixture but unstable when the activator and substrate are removed which, as described above, leads to different kinetic equations. It could also result from the fact that the extent to which the enzyme complexes $F_i S_2$ and $F_i I_2$ are being formed is unknown and they are not taken into consideration by this model. When the change which occurs when the inactive form of the modified enzyme becomes active has been determined this question will be better answered.

The lack of fit could also be due simply to excessive scatter in the data. The assay procedure could be altered to try to increase the accuracy and reduce this problem. One possible way of doing this would be by adding an equivalent amount of substrate along with the NaOH used as

titrant as Andersen did (1959a). This would allow larger concentrations of enzyme to be assayed without the problem of substrate depletion and thus greater slopes would be produced. The value of attempting to get more, and more accurate, kinetic data of this type is questionable until the basis of the activation is better understood. The mechanism is obviously complex and it seems that equations derived from such mechanisms, which involve reactions approaching equilibrium and concentration-dependent activation and inhibition, are of greater interest for their theoretical value and the qualitative predictions which can be made from them than for their practical applicability.

The problem might better be approached by trying to determine what the difference is between the active and inactive forms of the modified enzyme. If this was known, then direct measurement of this change or of a property related to this change may give more accurate data regarding the rate at which it occurs as it could be more easily isolated from other factors which affect the catalytic activity. For instance, if the activation represented a conformational change which was observable either directly in its fluorescence or circular dichroism spectra and a substrate was known that could be used in the activation without interfering with the measurement, or through an alteration in the enzyme's reaction with a specific reagent, then this property could be measured directly. Such an approach might allow this one reaction step to be studied apart from the rest of the reaction mechanism and therefore would clarify what is happening.

6. ENZYME STRUCTURE

6.1 INTRODUCTION

The kinetic behaviour of sulphatase A has led some authors to postulate that a conformational change may be associated with or responsible for the formation and/or activation of the modified enzyme. In particular this is suggested by the fact that the modified and native forms of the enzyme have such different kinetic characteristics and yet the modified enzyme can easily revert to its original state. Lee and Van Etten (1975a) measured the temperature dependent reversion with the rabbit liver enzyme and calculated the entropy of activation to be -8 cal $mole^{-1}$ deg, a value which would be consistent with a process involving a small conformational change but not with one involving any major disruption of the structure. Later Rybarska-Stylinska and Van Etten (1979) prepared antiserum to the native rabbit liver sulphatase A and reacted it with both the native and substrate-modified forms. The native enzyme bound 5-7 IgG molecules per monomer while the modified enzyme bound 3-4 IgG per monomer. These results suggest the occurrence of either significant conformational changes or covalent modification of residues at the antigenic sites, or both.

The activation of the modified enzyme may also involve a conformational change. The hypothesis that the modified enzyme has two binding sites and the native enzyme only one requires that the second site become accessible before the modified enzyme can be activated. Refolding of part of the

molecule or a slow isomerization may be responsible for this accessibility and could explain the slow rate of activation. Since preincubation with the activator does not increase the rate of activation the rate determining step is not simply the slow binding of sulphate. The presence of the unaltered modified enzyme after sulphate activation indicates that a structural change has not occurred and that the activator has not been irreversibly bound. This all suggests a slight, easily reversible, difference between the modified and native forms of the enzyme which does not affect its stability to any great extent and would be consistent with conformational motion.

If such changes are occurring but are very localized they may be detectable only through changes in the catalytic activity of the enzyme or perhaps through differences in the rate of inactivation of the two forms by proteolytic enzymes or denaturing agents. If, however, they are more substantial and involve large areas of the molecule or many amino acid residues they may be detected using optical techniques. Ultraviolet absorption, fluorescence and circular dichroism spectra were taken of the native and modified forms of the ox liver enzyme to see if such a change could be observed. Spectra of the activated modified enzyme were not taken because of difficulties in obtaining a preparation which was sufficiently concentrated for such spectral studies, and because kinetic evidence suggested that the activated form of the enzyme was unstable and could not be isolated as such. As described in Chapter 4, the enzyme isolated after sulphate activation had the kinetic characteristics of a mixture of

native and modified enzyme similar to that produced on heating the modified enzyme for 1 hour at 45°C. A sample of the modified enzyme was treated in this way and the CD spectra of this solution did not differ from that of either the native or modified enzyme, as shown below. Changes which might be occurring during activation could not be studied as this is observed only in the presence of substrate. Since all of the aryl sulphates used as substrates absorb light and would interfere with optical measurements and because changes in the concentrations of substrate, product, sulphate and the different forms of enzyme could not be eliminated under these conditions it was not possible to study this stage of the reaction.

6.2 ENZYME SOLUTIONS

Samples of both the native and two independent preparations of the modified enzyme were dialyzed against 0.1 M Tris-HCl, pH 7.4, I=0.1 or 0.07 M sodium acetate buffer pH 5.0, I=0.1 for three days. These buffers were then used as blanks in the spectral studies. All solutions were filtered through a 0.45 μm millipore filter before the spectra were taken. The concentration of the enzyme was approximately 0.6 mg ml^{-1} for the CD and UV spectra and 0.1 mg ml^{-1} for the fluorescence spectra. The enzyme activity of the solutions used was measured both immediately before the CD spectra were taken and after to ensure that the spectra did not reflect any unexpected alteration of the enzyme structure. The activity was again checked after the UV and fluorescence spectra were taken. The values obtained are given in Table 22 and show no change in activity over the time the spectra were measured.

TABLE 22

INITIAL VELOCITIES OF ENZYME SOLUTIONS MEASURED WITH NITROCATECHOL SULPHATE

Enzyme Solution	v_o before Spectral studies	v_o after CD spectra	v_o after UV and Fluorescence spectra
	μmoles $min^{-1}mg^{-1}$	μmoles $min^{-1}mg^{-1}$	μmoles $min^{-1}mg^{-1}$
native pH 7.4	204	204	208
native pH 5.0	204	202	204
modified pH 7.4	33	31	36
	25	28	30
modified pH 5.0	34	33	36
	25	28	28
modified pH 5.0 heated 45°/1hr	87	87	-

6.3 SEDIMENTATION EQUILIBRIUM CENTRIFUGATION

Sulphatases A from several sources have been clearly shown to exist in different polymeric forms. Although not studied extensively in most cases, the pH of the solution appears to be the predominant factor governing the self-association. The more detailed studies carried out with the ox liver and rabbit liver enzymes demonstrate that ionic strength and temperature also have an effect. For example, the enzyme isolated from sheep brain (Balasubramanian and Bachhawat, 1975) was shown by gel filtration to exist as a monomer of molecular weight 122 000 at pH 7.5 and to polymerize at pH 5.0. Similarly the molecular weight of rat liver sulphatase A is 130 000 at pH 7.5, I=0.1 and 400 000 at pH 5.0, I = 0.1 (Worwood *et al*, 1973) and that of

rabbit testis is 110 000 at pH 7.1 and 220 000 at pH 5.0 (Yang and Strivastava, 1976). Sedimentation equilibrium was used by Draper *et al*, (1976) to determine the molecular weight of the human liver enzyme. A value of 104 500 was obtained at pH 8.1 and of 413 000 at pH 5.0 with no heterogeneity observed at either pH. Nichol and Roy (1965) and Jerfy *et al*, (1976) have established that ox liver sulphatase A can form polymers containing up to four monomeric units. It exists predominantly as a monomer above pH 6.5 at I=0.1 but forms a tetramer at pH's below 5.5 at this ionic strength if the enzyme concentration is greater than 5 $\mu g\ ml^{-1}$. A single peak was observed by Nichol and Roy (1965) using sedimentation velocity ultracentrifugation at pH 7.5 and I=0.2 which corresponded to a dimer, whereas at pH 6.3, I=0.1 the dimer coexisted in rapid equilibrium with monomer, trimer and tetramer in the concentration range 0.1-1.0%. Similarly Waheed and Van Etten (1979) have shown that the rabbit liver enzyme exists predominantly as a monomer at pH 7.5 and a dimer at pH 4.5 with an ionic strength of 0.1. If the ionic strength is increased to 1.0 no difference was observed at pH 4.5 but dissociation tends to occur at pH 5.2. They proposed that because the dimer does not exhibit the anomalous kinetics and would be the dominant form present under physiological conditions, the dissociation of the enzyme under experimental conditions was responsible for the substrate-induced inactivation. Work done with the red kangaroo liver enzyme showed that while the kinetic properties of the enzyme were analogous to those observed with the ox liver enzyme, no polymerization was evident under similar conditions (Roy, 1971a). This, along with the fact that inactivation is

not concentration dependent (Nicholls and Roy, 1971) and that an enzyme-antibody complex which cannot polymerize also does not exhibit the anomalous kinetics at pH 4.5 (Rybarska-Stylinska and Van Etten, 1979), indicates that the extent of association does not affect the modification reaction. This has been confirmed for the ox liver enzyme (Roy, 1978) which was shown to exhibit the same kinetic properties regardless of the dominant species present. It is possible, however, that the polymerization pattern observed with the modified enzyme might differ from that of the native enzyme if the net charge on the enzyme monomer was altered during inactivation.

6.31 Methods

Sedimentation equilibrium was used to determine if the polymerization pattern of the modified enzyme was different from that of the native enzyme. The Yphantis meniscus depletion method (Yphantis, 1964) was chosen as it eliminates the need to accurately determine the initial concentration of the solution and requires only small quantities of enzyme. The runs were done at 5°C because the modified enzyme reverts back to the native form with a $t_{\frac{1}{2}}$ of 60 hours at 20°C and pH 7.4. As these runs took up to 33 hours this would have meant an appreciable percentage of the native enzyme being reformed during the course of the experiment.

In this method a running speed is calculated such that the protein concentration at the meniscus falls to zero and a region of straight fringes is produced. It is recommended by Yphantis that σ, the effective reduced

molecular weight, be set equal to 5 cm^{-1} to achieve this when 3 mm high columns are used. The effective reduced molecular weight is defined by Yphantis and Waugh (1956) as

$$\sigma = \frac{\omega^2 M(1-\bar{v}\rho)_o}{RT}$$

where $(1-\bar{v}\rho)_o$ is evaluated at infinite dilution and at a pressure of 1 atm. For sulphatase A at pH 7.4 where M = 107 000 a speed of 18 000 rev/min is required and at pH 5.0 where M = 430 000 a speed of 10 000 rev/min is required.

The time to reach equilibrium was calculated from the equation

$$t_{eq} = \frac{2.3(b-a)}{\omega^2 sr}$$

This will give the time required for the concentration difference across the cell, c(b)-c(a), to be within a fraction, $\varepsilon = 10^{-3}$, of its equilibrium value. It predicts a time of 27 hours to reach equilibrium at pH 7.5 at 5°C, 33 hours at pH 5.0 at 5°C and 27 hours at pH 5.0 at 20°C. As an experimental check two photographs were taken and measured for each run approximately 2 hours apart before the centrifuge was stopped. No difference was detected between these photographs for any of the experiments.

A Spinco Model E analytical ultracentrifuge was used with a RTIC unit to control and measure the temperature and Rayleigh interference optics. The optics were set up with a Depil filter having $\lambda_{max} = 547$ nm and 1 mm

symmetrical parallel slits. An An-D rotor and a double sector aluminium filled-epoxy cell with sapphire windows and Kel-F liners were used. The reference side of the cell contained 0.1 ml Kel-F oil and 0.12 ml buffer and the other side 0.1 ml Kel-f oil and 0.11 ml enzyme solution. Slightly more buffer was used to ensure the entire column length of the solution to be measured was visible. The enzyme solutions, of approximately 0.6 mg ml^{-1}, were dialyzed at 5°C for 3 days against either veronal buffer, pH 7.5, I=0.1 or sodium acetate buffer, pH 5.0, I=0.1. The enzyme activity of these solutions had been determined by pH-stat assay before analysis. The actual running times were; 18 000 rev/min for 27 hours at pH 7.5 and 5°C, 10 000 rev/min for 33 hours at pH 5.0 and 5°C, 10 000 rev/min for 23 hours at pH 5.0 and 20°C.

The molecular weight was determined from the displacement of the fringes down the length of the column relative to their position at the meniscus. A Nikon profile projector Model 6C with Nikon reversible counter ERC-M-251 was used to measure the photographic plates. Measurements were made on five successive fringes, three white and two dark, usually at 0.5 mm intervals near the meniscus and at 0.1 mm intervals once the fringe displacement had become approximately 20 μ. With the runs at pH 5.0 where it was attempted to calculate an association constant readings were taken at 0.1 mm intervals for the entire length of the fringes. Readings were always taken using the centre fringes to reduce error and increase the number of readings which could be obtained. The fringe width was

285 μ and the scatter in the 5 readings was 1 μ.

The five readings at each point along the cell (r) were averaged to give the displacement (y). The first three to five y values were then averaged to give y_o, the reference value. With several runs photographs were taken immediately after the required speed had been reached to correct for any deviation of the fringes resulting from imperfections in the optical system or cell windows. These plates were measured in the same way and the displacement value at each interval subtracted from the displacement obtained at the end of the experiment. Correction with this base line made no difference to the molecular weight calculated and therefore it was not always done.

The displacement values at given distances from the centre of rotation, y(r), are then plotted as $\ln[y(r)-y_o]$ versus $[r^2/2]$. The slope of this line gives the weight-average effective reduced molecular weight which can be converted to the weight-average molecular weight by multiplying by $RT/(1-\bar{\upsilon}\rho)\omega^2$. This calculation was done using chained programs on a PDP8 computer using the partial specific volume of 0.715 determined by Nichol and Roy (1965) for native sulphatase A. The sets of five readings were fed directly into the program and the values of $\ln[y(r)-y_o]$ across the cell were calculated and plotted by a Hewlett-Packard 7200A/7202A graphic plotter. The slope of this line and point slopes at a selected interval across the cell were calculated.

The data obtained at pH 5.0 at 5°C was used to determine an association constant for the monomer-tetramer system by the method of Milthorpe, Jeffrey and Nichol (1975). This

involves converting the displacement values to concentrations and making a plot of $\bar{c}(r)$, the total concentration of protein at point r, versus r. The function $\Omega(r)$ is then calculated as

$$\Omega(r) = \frac{\bar{c}(r)\ \exp(\phi_m M_m\ (r_F^2 - r^2)}{\bar{c}(r_F)}$$

$$\phi_m = \frac{(1-\bar{\upsilon}_m \rho)\omega^2}{2RT}$$

where

M_m = molecular weight monomer

$\bar{\upsilon}_m$ = partial specific volume monomer

ρ = density of solution

R = gas constant

T = temperature of the experiment

A plot of $\Omega(r)$ versus $\bar{c}(r)$ is then made and a value $\Omega^\circ(r)$ obtained by extrapolation to zero concentration. The activity of the monomer can now be calculated at each distance r from the relationship

$$a_m(r) = \bar{c}(r)\ \frac{\Omega^\circ(r)}{\Omega(r)}$$

and subsequently K_{ass} for a monomer-tetramer system as

$$\frac{\bar{c}(r) - a_1(r)}{[a_1(r)]^4} = K_{ass}$$

6.32 Results and Discussion

The values obtained for the molecular weight of sulphatase A under the different conditions are given in Table 23. The results agree with those found by Nichol and Roy (1965) for native sulphatase A in that the enzyme exists as a single molecular weight species at pH 7.5 of weight approximately 110 000 and primarily as a species of molecular weight 430 000 at pH 5.0.

With the modified enzyme at pH 5.0 and 5°C some dissociation was detected as a decrease in the point-average molecular weight at low enzyme concentration which can be seen in Figure 23. This had not been found with the native enzyme at pH 5.0 and 20°C by Nichol and Roy. Equilibrium runs with the native enzyme at pH 5.0 at 20°C and 5°C were carried out to verify this difference. At 20°C a single molecular weight species was found as before but at 5°C the presence of some lower molecular weight material was again detected suggesting that some dissociation was occurring. Association constants were calculated as described above assuming a monomer tetramer system, and values of 0.2×10^6 $l^3 g^{-3}$ for the modified enzyme and 1.0×10^6 $l^3 g^{-3}$ for the native enzyme were found. Some question surrounds the accuracy of these values as the extrapolation to $\Omega^\circ(r)$ is uncertain. The method requires that a sufficiently high concentration of the lower molecular weight form is present to allow an accurate extrapolation back to zero concentration. This was not the case here where dissociation is slight and the scatter in the data, resulting primarily from the small fringe displacements made it difficult to choose the best

TABLE 23

SEDIMENTATION EQUILIBRIUM

Conditions		Initial Concentration (g/100 ml)	Molecular Weight
native enzyme pH 7.5, 20°	19,000 rpm 20 hr	0.1	106,000
		0.01	123,000 (Nichol, Roy 1965)
modified enzyme pH 7.5, 5°	20,000 rpm 25 hr	0.07	115,000
	18,000 rpm 27 hr	0.06	120,000
native enzyme pH 5.0, 5°	10,000 rpm 33 hr	0.07	430,000 (dissociation)
modified enzyme pH 5.0, 5°	10,000 rpm 31 hr	0.07	440,000 (dissociation)
	10,000 rpm 33 hr	0.06	414,000 (dissociation)

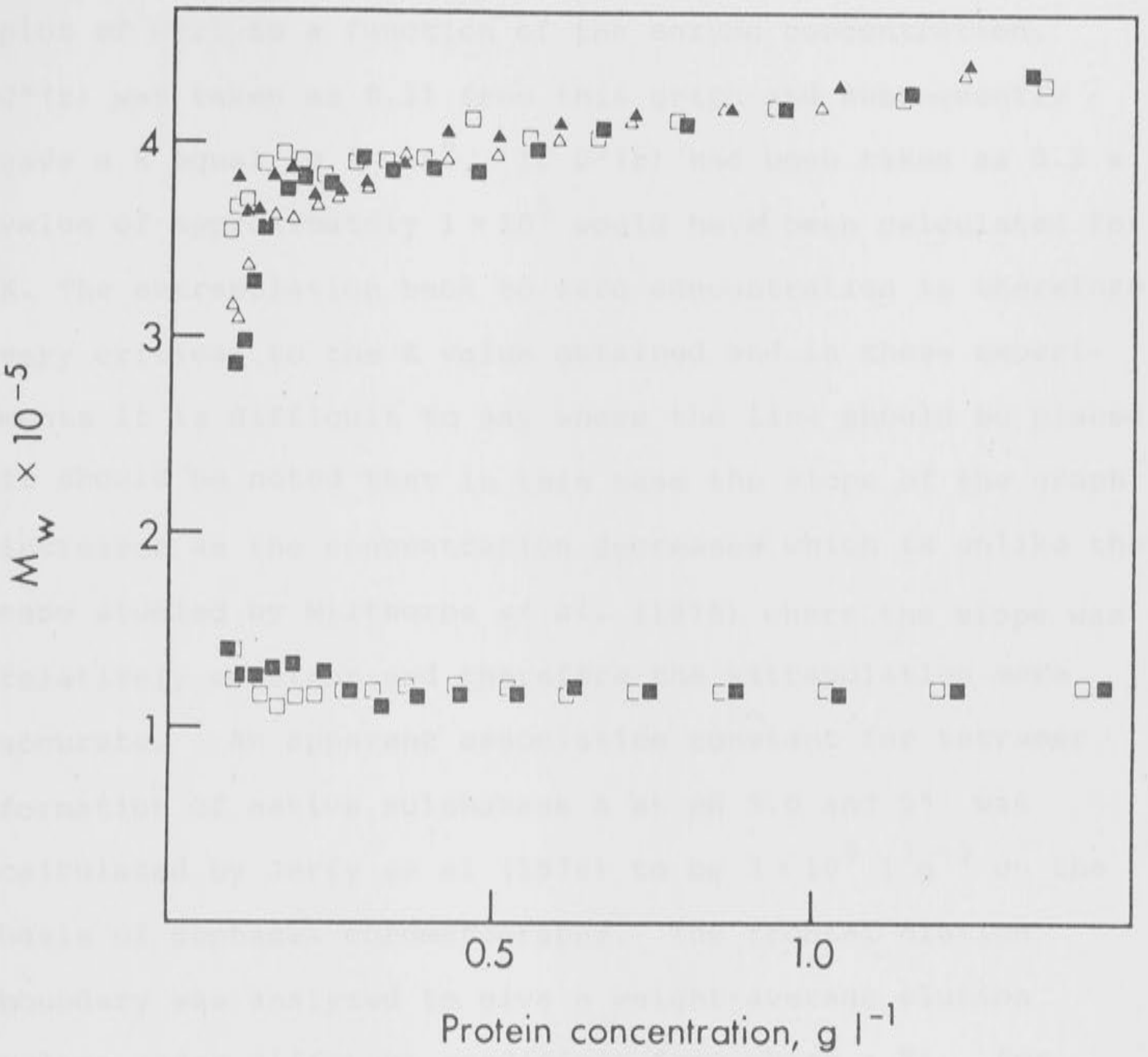


Figure 23 Point-average values of the weight-average molecular weights of native and substrate-modified sulphatase A as a function of the total protein concentration during equilibrium ultracentrifugation at 5°C, I=0.1.

□ , ■ ; upper curve, substrate-modified sulphatase A after centrifugation for 31 and 33 hours, respectively, at pH 5.0; lower curve, substrate-modified sulphatase A after 25 and 27 hours centrifugation at pH 6.5. △ , ▲ ; native sulphatase A after centrifugation at pH 5.0 for 31 and 33 hours.

fitting line. This can be seen in Figure 24 which is a plot of $\Omega(r)$ as a function of the enzyme concentration. $\Omega°(r)$ was taken as 0.11 from this graph and subsequently gave a K equal to 1×10^6. If $\Omega°(r)$ had been taken as 0.2 a value of approximately 1×10^5 would have been calculated for K. The extrapolation back to zero concentration is therefore very critical to the K value obtained and in these experiments it is difficult to say where the line should be placed. It should be noted that in this case the slope of the graph increases as the concentration decreases which is unlike the case studied by Milthorpe *et al*, (1975) where the slope was relatively constant and therefore the extrapolation more accurate. An apparent association constant for tetramer formation of native sulphatase A at pH 5.0 and 5° was calculated by Jerfy *et al* (1976) to be 3×10^9 $l^3 g^{-3}$ on the basis of Sephadex chromatography. The frontal elution boundary was analyzed to give a weight-average elution volume under different conditions from which a K_{app} for association could be derived. The much less concentrated enzyme solutions used in this determination (0.5-50 mg l^{-1}) compared to those used in the sedimentation equilibrium experiments (0.1-1.0 g l^{-1}) may account for the difference between the two apparent association constants. The association constant obtained from the chromatography data was calculated assuming that the system contained a monomer and tetramer only. This simplification may become increasingly invalid at higher protein concentrations. The method of Milthorpe *et al*, (1975) does not require this simplification but, as described above, cannot properly

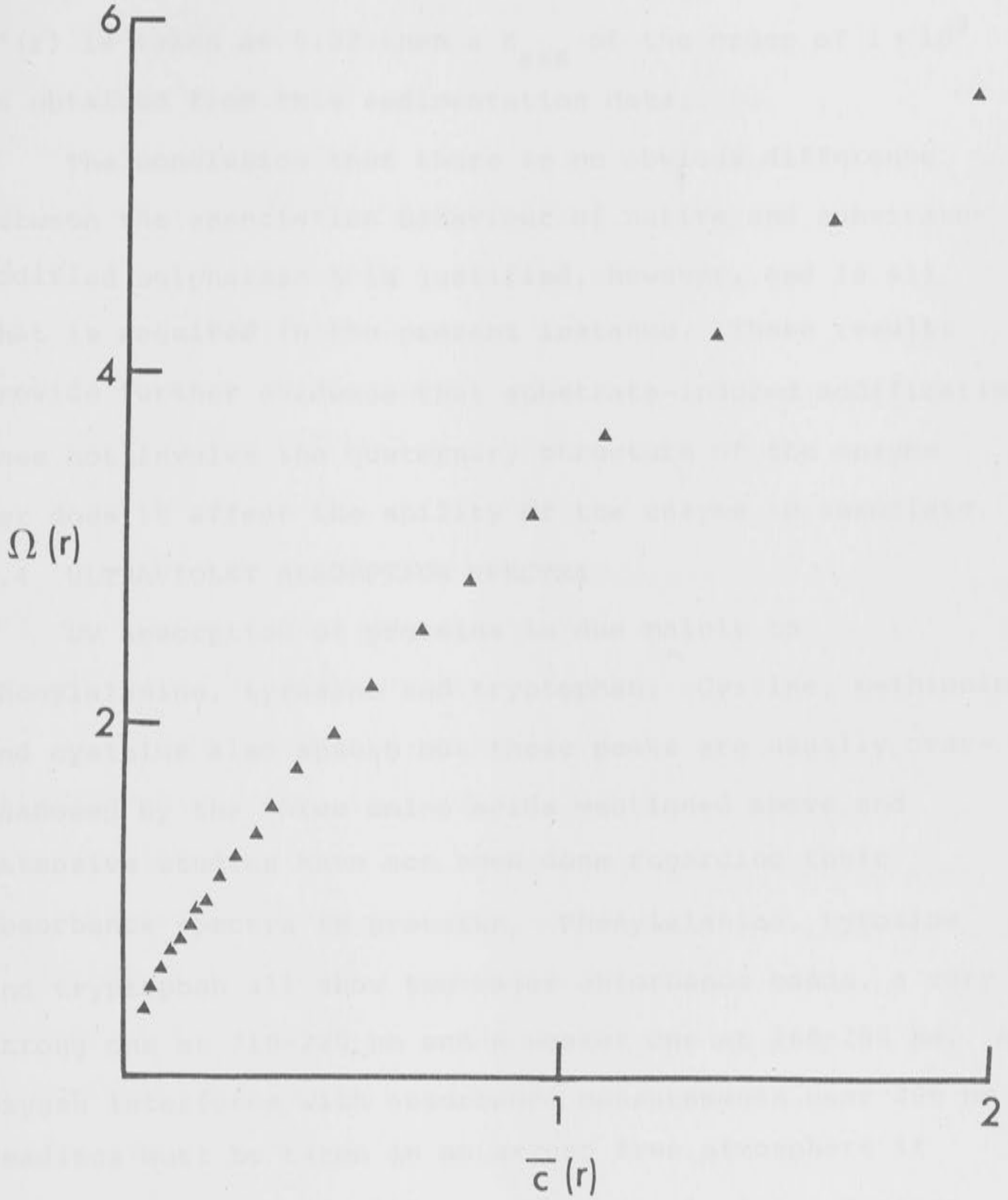


Figure 24 Plot of the function Ω(r), defined in the text, versus the protein concentration at different positions in the cell. Extrapolation of the curve to zero concentration gives Ω°(r) which is required to calculate the association constant.

handle the sulphatase A system. It should be noted that if $\Omega°(r)$ is taken as 0.02 then a K_{ass} of the order of 1×10^9 is obtained from this sedimentation data.

The conclusion that there is no obvious difference between the association behaviour of native and substrate-modified sulphatase A is justified, however, and is all that is required in the present instance. These results provide further evidence that substrate-induced modification does not involve the quaternary structure of the enzyme nor does it affect the ability of the enzyme to associate.

6.4 ULTRAVIOLET ABSORPTION SPECTRA

UV absorption of proteins is due mainly to phenylalanine, tyrosine and tryptophan. Cystine, methionine and cysteine also absorb but these peaks are usually over-shadowed by the three amino acids mentioned above and extensive studies have not been done regarding their absorbance spectra in proteins. Phenylalanine, tyrosine and tryptophan all show two major absorbance bands, a very strong one at 210-220 nm and a weaker one at 260-280 nm. As oxygen interferes with absorbance measurements near 200 nm readings must be taken in an oxygen free atmosphere if values obtained below 220 are to be meaningful. Because the peptide bond contributes substantially to the region below 250 nm and it is very unlikely that the modification would affect the primary structure of the enzyme it was not attempted to measure accurately the absorbance below 220 nm. The peak at 280 is believed to represent similar $\pi \rightarrow \pi^*$ transitions in all of the three chromophores, the strongest absorber being tryptophan.

Either a shift in the position of the absorption bands or a change in their intensity might be expected if a measurable conformational change has occurred. Such a change in the spectrum of a protein is related to changes in the ionization of sensitive residues, in their exposure to the solvent, or to other immediate environmental effects. For example, spectra have been shown to alter as a result of ionization of groups adjacent to the phenolic group of a tyrosine residue (Wetlaufer *et al*, 1958) and of alteration to the hydrogen bonding of the tyrosyl hydroxyls (Perlmann *et al*, 1960). Thus if there was a conformational difference between the two forms of sulphatase A or if the binding of a substrate or effector molecule had changed the environment of chromophores present at or near the active site a difference might have been detected in their spectra. One documented case of this occurring is with the binding of glycol chitin to lysozyme. This produces a red shift in the absorption of an indole chromophore which was shown to result from one of four solvent-accessible indole chromophores of the lysozyme molecule being buried as a consequence of substrate binding (Hayachi *et al*, 1963). When the binding sites are saturated $\Delta\varepsilon_{293.5} = -1550$ which can be accounted for by the transfer of a single indole chromophore from the solvent water to the protein interior. Similarly changes in the spectra of α-chymotrypsin on acylation are thought to result from the formation of an internal cross-linkage between an amino acid residue and the indole ring of a tryptophan residue or from transfer of one or more tryptophan residues to a hydrophobic region of the molecule

(Wootton and Hess, 1962).

6.41 Method

The UV absorbance of the native and modified sulphatase A solutions was measured between 200 and 350 nm at room temperature with a Varian SuperScan 3 spectrophotometer. The solutions were scanned above 300 nm to ensure that there was no turbidity present which could not be detected by eye as no protein absorption would be expected in this region.

6.42 Results and Discussion

Figure 25 shows the spectra obtained with the native and modified enzymes at pH 7.4 and pH 5.0. No difference between the two forms of enzyme could be detected at either pH. The only difference observed was a slight increase in absorbance in the region of 250-280 nm at pH 5.0 over that seen at pH 7.4, but this was found with both native and modified enzyme. Apart from the major peak at 280 nm ripples are evident in the curve between 250 and 280 nm which are generally believed to result from fine structure in the protein. More specifically small peaks are observed at 259 and 265 nm and slight ripples at 253 and 271 nm. These wavelengths correspond to those given by Beaven and Holiday (1952) for phenylalanine absorption although they also note a small peak at 248 nm which is not noticeable here. Because of the low extinction coefficient of phenylalanine compared to other chromophores, these peaks are not often visible in protein spectra and it is probably only the fact that there are 50 residues in sulphatase A which makes its contribution observable. The strongest peak is at 259 nm and can be correlated to

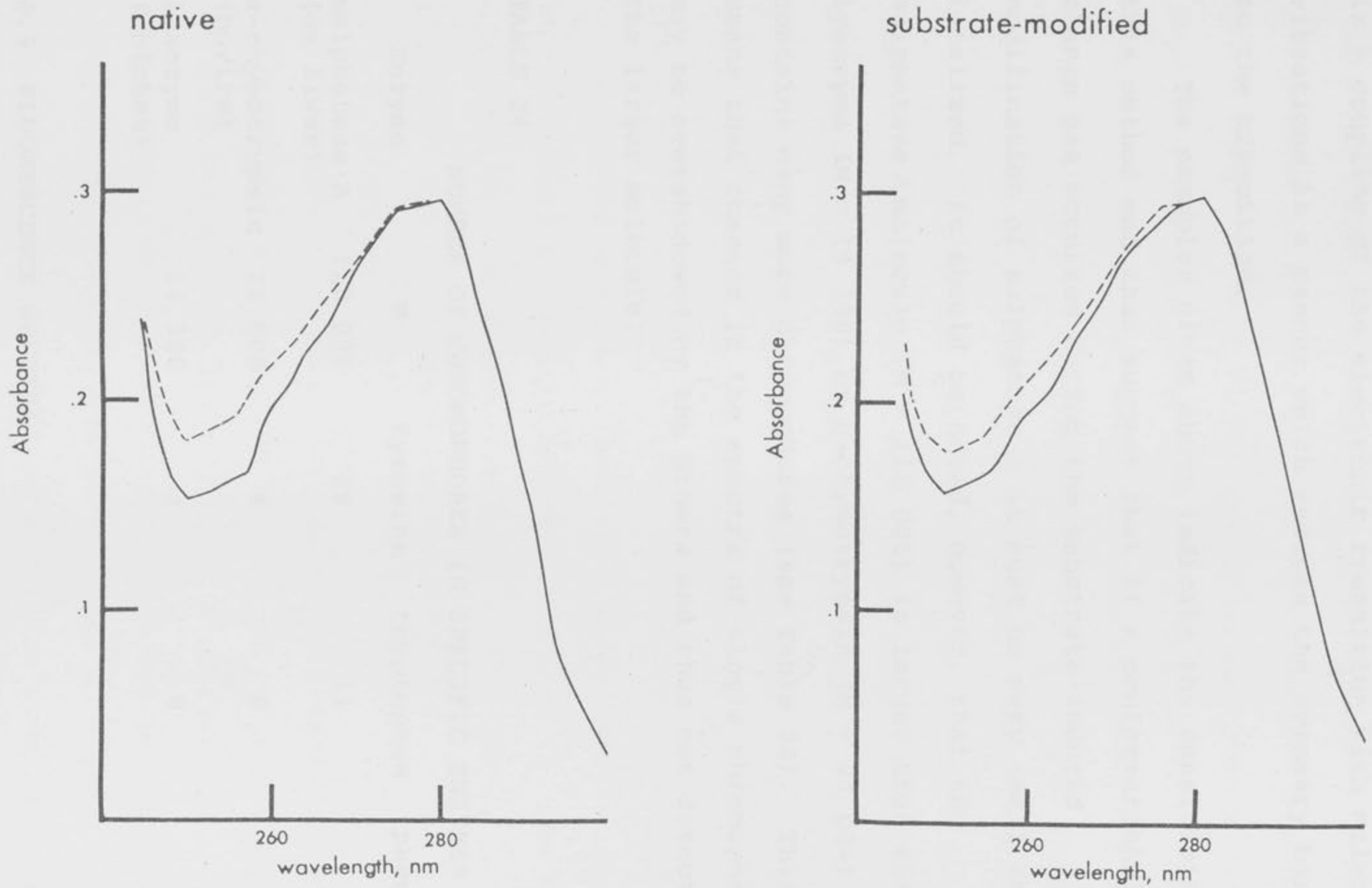


Figure 25 The ultra-violet absorption spectra of native and substrate-modified sulphatase A at room temperature, in 5 mM Tris-HCl, pH 7.4, I=0.1 (———) and in 0.07 M sodium acetate buffer, pH 5.0, I=0.1 (----).

an absorbance of phenylalanine which corresponds to a forbidden $\pi \rightarrow \pi^*$ transition known to be able to occur if there is a coupling of the electronic transition with molecular vibrations in a manner which reduces the symmetry barrier to the transition.

The examples given above indicate the sensitivity of this method and thus suggest that if a conformational change has occurred during the substrate-induced modification of sulphatase A it must be very small or localized. It should be noted, however, that the sulphatase A molecule (M = 110 000) is larger than either lysozyme (M = 14 300) or α-chymotrypsin (M = 24 000) and contains many more chromophores (see Table 24). This means that changes in the spectra of single chromophores may be overshadowed by the others and thus not detected in the larger molecule.

TABLE 24

NUMBER OF CHROMOPHORES IN SPECIFIC ENZYMES

Enzyme	M	tyrosine	tryptophan	phenylalanine
sulphatase A (ox liver)	110 000	28	11	50
α-chymotrypsin (bovine)	24 000	4	8	6
lysozyme (chicken)	14 300	3	6	3

6.5 FLUORESCENCE SPECTRA

Fluorescence spectra are similar to UV absorption spectra in that they are sensitive to changes in the immediate environment of the chromophores and have been

found to be a convenient measure of localized changes in conformation where a chromophore is involved. An example where the fluorescence spectrum of an enzyme is altered by the binding of a ligand is beef heart lactate dehydrogenase. The emission of this enzyme at 340 nm was quenched when DPNH or 3-acetylpyridine-DPNH was bound (Shifrin and Kaplan 1958; Shifrin *et al*, 1959). In studies involving larger enzymes the use of fluorescent probes to detect conformational change has often proven successful where the changes are too slight to be observed in the intrinsic fluorescence spectra of the protein. Such is the case with glycogen phosphorylase b, an enzyme having a molecular weight of 195 000. The environment in the region of the binding site for 1-anilino-naphthalene-8-sulphonate (the fluorescent probe) was shown to be altered on the binding of caffeine, an allosteric inhibitor, such that a decrease in the quantum yield of fluorescence and a change in spectral distribution was observed (Steiner *et al*, 1980). A suitable probe has yet to be found for sulphatase A.

6.51 Method

Fluorescence spectra were measured manually at excitation wavelengths of 275, 280 and 290 nm recording the emission between 200 and 500 nm at 5 nm intervals. An Aminco-Bowman spectrofluorimeter was used with two 3/16" slits and the photomultiplier slit set to either 0.5 or 2 mm. Solutions of different concentrations were used to check for any inner filter effect resulting from the reabsorption of emitted light. Identical spectra were also taken of the buffers and subtracted from those containing

enzyme in order to eliminate light scattering contributions.

6.52 Results and Discussion

As with the UV spectra no difference was detected between the native and modified enzyme at either pH 7.4 or pH 5.0. All spectra showed a single major peak at λ_{em} = 325 nm which is characteristic of tryptophan fluorescence and a smaller peak at λ_{em} = 270 nm due to phenylalanine when λ_{ex} = 275 nm. The spectra of the substrate-modified enzyme are shown in Figures 26 and 27 for λ_{ex} = 275 nm and 290 nm.

These results are not unexpected as tryptophan fluorescence has been found to dominate the spectra of the vast majority of proteins. It generally overshadows any phenylalanine fluorescence because of its much higher quantum yield. Chen (1967) gave values of 0.13, 0.14 and 0.024 for the quantum yields of tryptophan, tyrosine and phenylalanine in water using quinine as standard. Although the quantum yield of free tryptophan and tyrosine are very similar λ_{em} for tyrosine occurs on the slope of the tryptophan fluorescence peak and therefore can be difficult to detect if the intensity of the tyrosine fluorescence is much less than that due to tryptophan. Also tyrosine is very susceptible to quenching and its quantum yield in proteins is generally in the range 0.01-0.03. Many factors contribute to this quenching. The emission peak of tyrosine occurs at 300-305 nm which is overlapped by the absorption peak of tryptophan. This allows an energy transfer of the radiation produced by the fluorescence of tyrosine to the tryptophan residues nearby which means

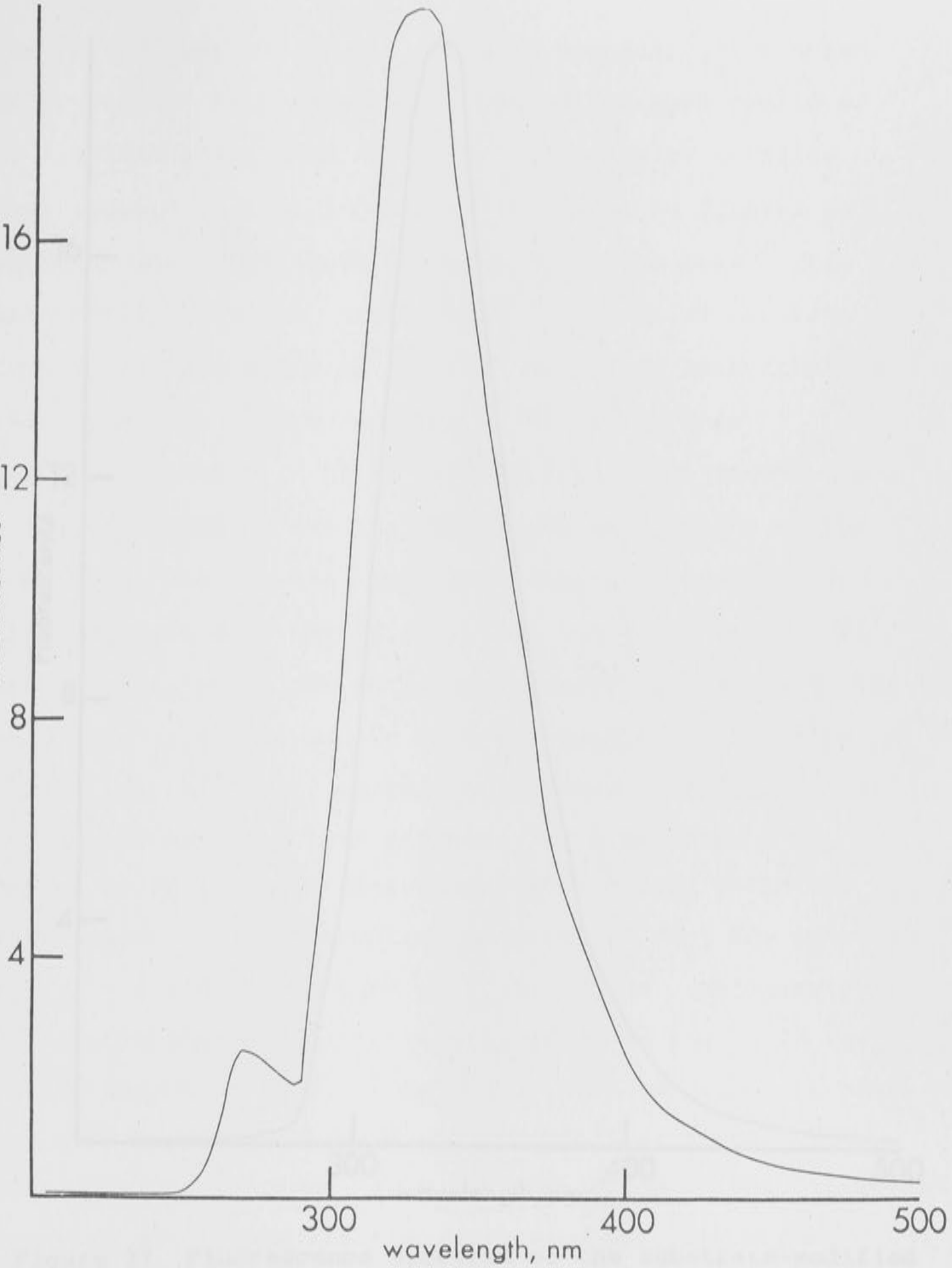


Figure 26 Fluorescence spectrum of the substrate-modified sulphatase A (c=0.01%) in 5 mM Tris-HCl, pH 7.4, I=0.1 at room temperature. The excitation wavelength was 275 nm and the emission was scanned from 200 to 500 nm at 5 nm intervals. The fluorescence is given in instrument units and has been corrected for scatter.

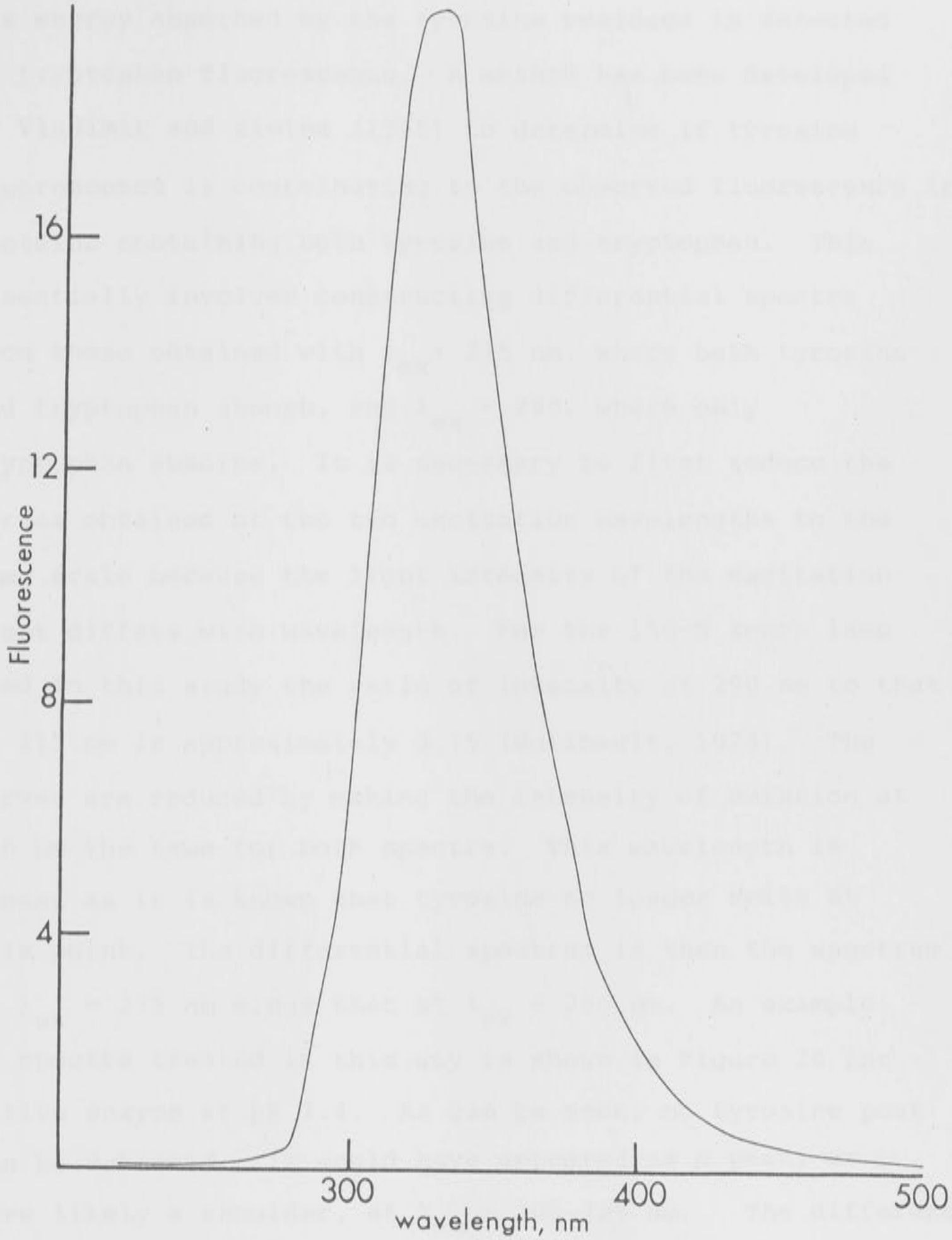


Figure 27 Fluorescence spectrum of the substrate-modified sulphatase A (c=0.01%) in 5 mM Tris-HCl, pH 7.4, I=0.1, at room temperature. The excitation wavelength was 290 nm and the emission was scanned from 200 to 500 nm at 5 nm intervals. The fluorescence is given in instrument units and has been corrected for scatter.

the energy absorbed by the tyrosine residues is detected as tryptophan fluorescence. A method has been developed by Vladimir and Zimina (1965) to determine if tyrosine fluorescence is contributing to the observed fluorescence in proteins containing both tyrosine and tryptophan. This essentially involves constructing differential spectra from those obtained with λ_{ex} = 275 nm, where both tyrosine and tryptophan absorb, and λ_{ex} = 290, where only tryptophan absorbs. It is necessary to first reduce the curves obtained at the two excitation wavelengths to the same scale because the light intensity of the excitation light differs with wavelength. For the 150-W Xenon lamp used in this study the ratio of intensity at 290 nm to that at 275 nm is approximately 3.75 (Guilbault, 1973). The curves are reduced by making the intensity of emission at 370 nm the same for both spectra. This wavelength is chosen as it is known that tyrosine no longer emits at this point. The differential spectrum is then the spectrum at λ_{ex} = 275 nm minus that at λ_{ex} = 290 nm. An example of spectra treated in this way is shown in Figure 28 for native enzyme at pH 7.4. As can be seen, no tyrosine peak can be detected. It would have appeared as a peak, or more likely a shoulder, at λ_{em} = 300-305 nm. The difference spectrum also shows the characteristic tryptophan peak with the small phenylalanine peak unchanged, as would be expected. This indicates that an energy transfer is occurring between tyrosine and tryptophan residues and that this accounts for much of the observed tryptophan fluorescence. The lack of a tyrosine peak does not, however,

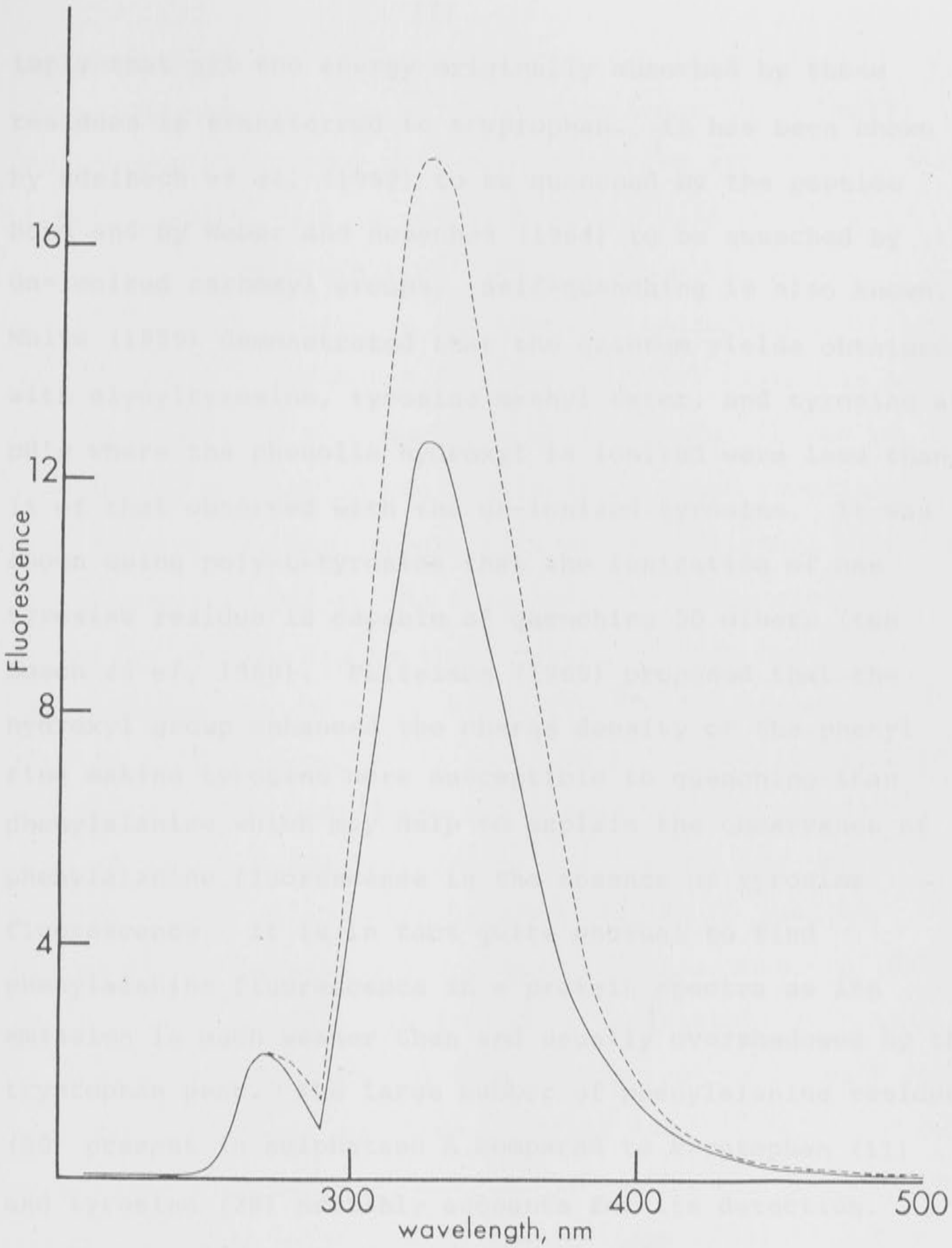


Figure 28 Difference spectrum for the native enzyme obtained by subtracting the spectrum produced with an excitation wavelength of 290 nm from that produced with an excitation wavelength of 275 nm. Spectra used for this calculation were those measured in 5 mM Tris-HCl, pH 7.4, I=0.1. The spectrum observed with λ_{ex}=275 nm is shown (----) as well as the difference spectrum (———).

imply that all the energy originally absorbed by these residues is transferred to tryptophan. It has been shown by Edelhoch *et al*, (1968) to be quenched by the peptide bond and by Weber and Rosenhek (1964) to be quenched by un-ionized carboxyl groups. Self-quenching is also known. White (1959) demonstrated that the quantum yields obtained with glycyltyrosine, tyrosine methyl ester, and tyrosine at pH's where the phenolic hydroxyl is ionized were less than 1% of that observed with the un-ionized tyrosine. It was shown using poly-L-tyrosine that the ionization of one tyrosine residue is capable of quenching 50 others (ten Bosch *et al*, 1968). Feitelson (1969) proposed that the hydroxyl group enhanced the charge density of the phenyl ring making tyrosine more susceptible to quenching than phenylalanine which may help to explain the observance of phenylalanine fluorescence in the absence of tyrosine fluorescence. It is in fact quite unusual to find phenylalanine fluorescence in a protein spectra as its emission is much weaker than and usually overshadowed by the tryptophan peak. The large number of phenylalanine residues (50) present in sulphatase A compared to tryptophan (11) and tyrosine (28) probably accounts for its detection.

The abundance of chromophores contributing to these spectra would indicate that a major disruption of the conformation, affecting the environment and thus emission of several residues would be required before a difference in the spectra would be detected.

6.6 CIRCULAR DICHROISM SPECTRA

Circular dichroism is more sensitive to changes in the

secondary structure of proteins than the other two spectral techniques used and can be used to estimate the α-helix, β-structure and random coil content of the structure. This property, which is a measurement of the different interaction of the molecule with left and right circularly polarized light, reflects the inherent conformation of that molecule, its orientation relative to other molecules and interactions with its immediate environment. It can be affected by changes of the magnitude of those resulting from raising the temperature of a protein solution from 3° to 25° as demonstrated by the transition of ribonuclease S-protein (Pflumn and Beychok, 1969). Such a change was also observed by Foster *et al*, (1976) in the spectrum of elastin. This spectrum was shown to be pH and temperature sensitive with an increased ellipticity of the positive band at 215 nm being observed at acid pH and low temperature. Changes in the ellipticity of the negative CD band at 350 nm of hen egg-white lysozyme with pH is observed (Nakae *et al*, 1972) as well. In this case the authors were able to show that this resulted from an interaction between two specific amino acid residues, Trp-108 and Glu-35, which had an intrinsic pK value of 6.9, and from the ionization of a tyrosyl residue which has an intrinsic pK of 10.18. The binding of ligands can also cause a change in an enzyme spectrum. For example, the CD spectra of the complexes of D-amino-acid oxidase with α-keto acids showed shifts of the component vibrational bands of the observed peaks with respect to the normal enzyme. The component band of the 450 nm transition showed a slight red shift while those

of the 380 nm band showed a blue shift. The degree of change paralleled the binding strength of the acid, with pyruvate causing less change than benzoate (Shiga and Shiga, 1973). Similarly Jaeneck (1970) noted an increase in the helicity of yeast glyceraldehyde-phosphate dehydrogenase on binding D-glyceraldehyde-3-phosphate.

These examples demonstrate the different ways in which the CD spectrum may change as a result of interaction with specific ligands, between different parts of the protein molecule, or with the solvent. Such a difference might be expected between the native and modified forms of sulphatase A.

6.61 Method

A Cary 60 spectropolarimeter was used with the cell-compartment temperature maintained at 10°C. The temperature was kept low to minimize reversion of the modified enzyme to its native form as the half-time for this reaction is approximately 5 hours at 37°C. A baseline was obtained by taking spectra of the dialysis buffers.

6.62 Results and Discussion

Figures 29 and 30 show the spectra of the modified and native enzymes at pH 7.4 and pH 5.0. Specific ellipticities are expressed in deg cm^2 $dmol^{-1}$ using a mean residue weight of 115 to calculate the mean residue molar concentrations. The mean residue rotation is given by

$$[\theta]_\lambda = \frac{r \times M_o}{l \times c \times 1000}$$

where

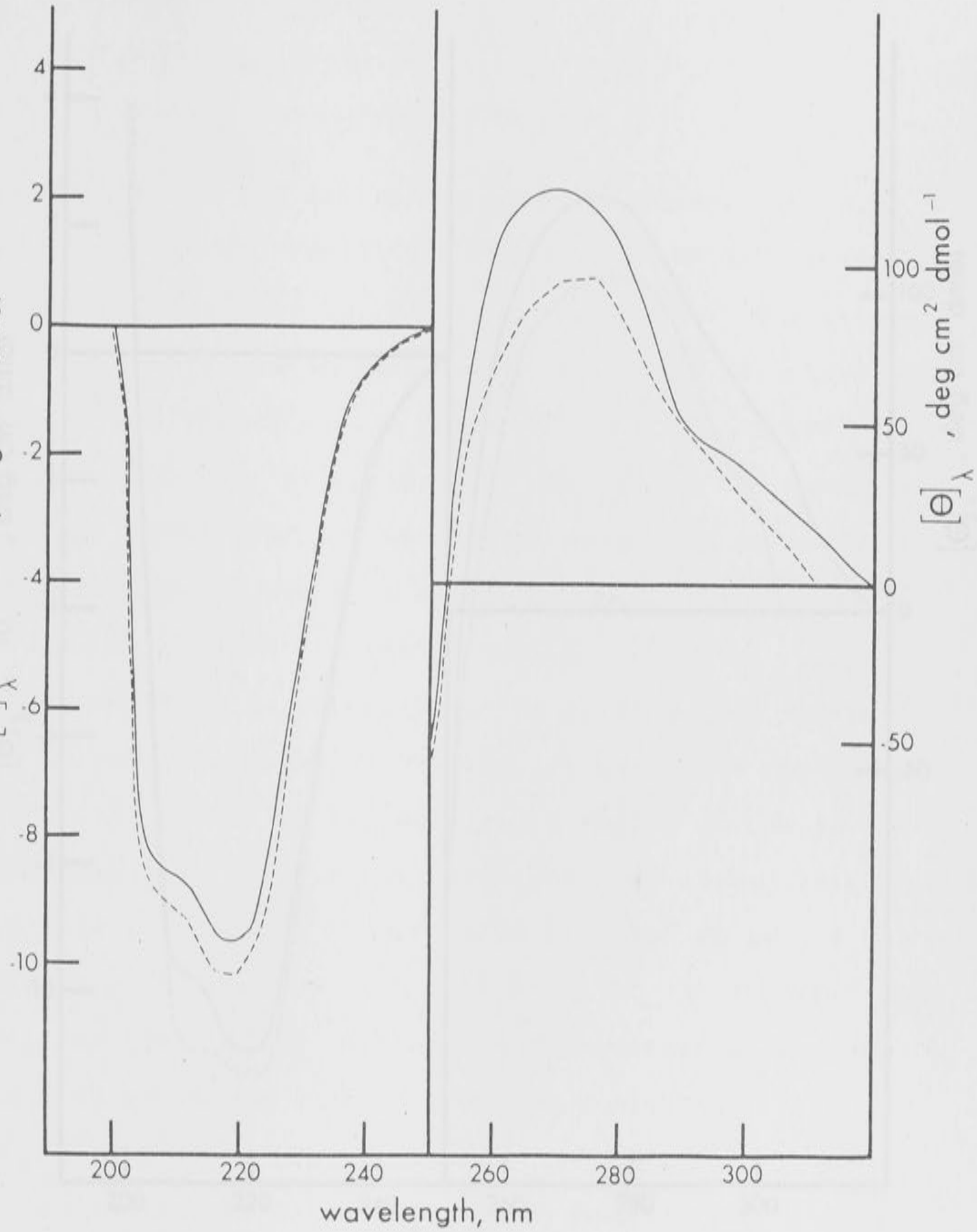


Figure 29 The aqueous circular dichroism spectra of native sulphatase A (c=0.06%) in 5 mM Tris-HCl, pH 7.4, I=0.1, (——), and in 0.07 M sodium acetate buffer, pH 5.0, I=0.1, (----), at 10°C.

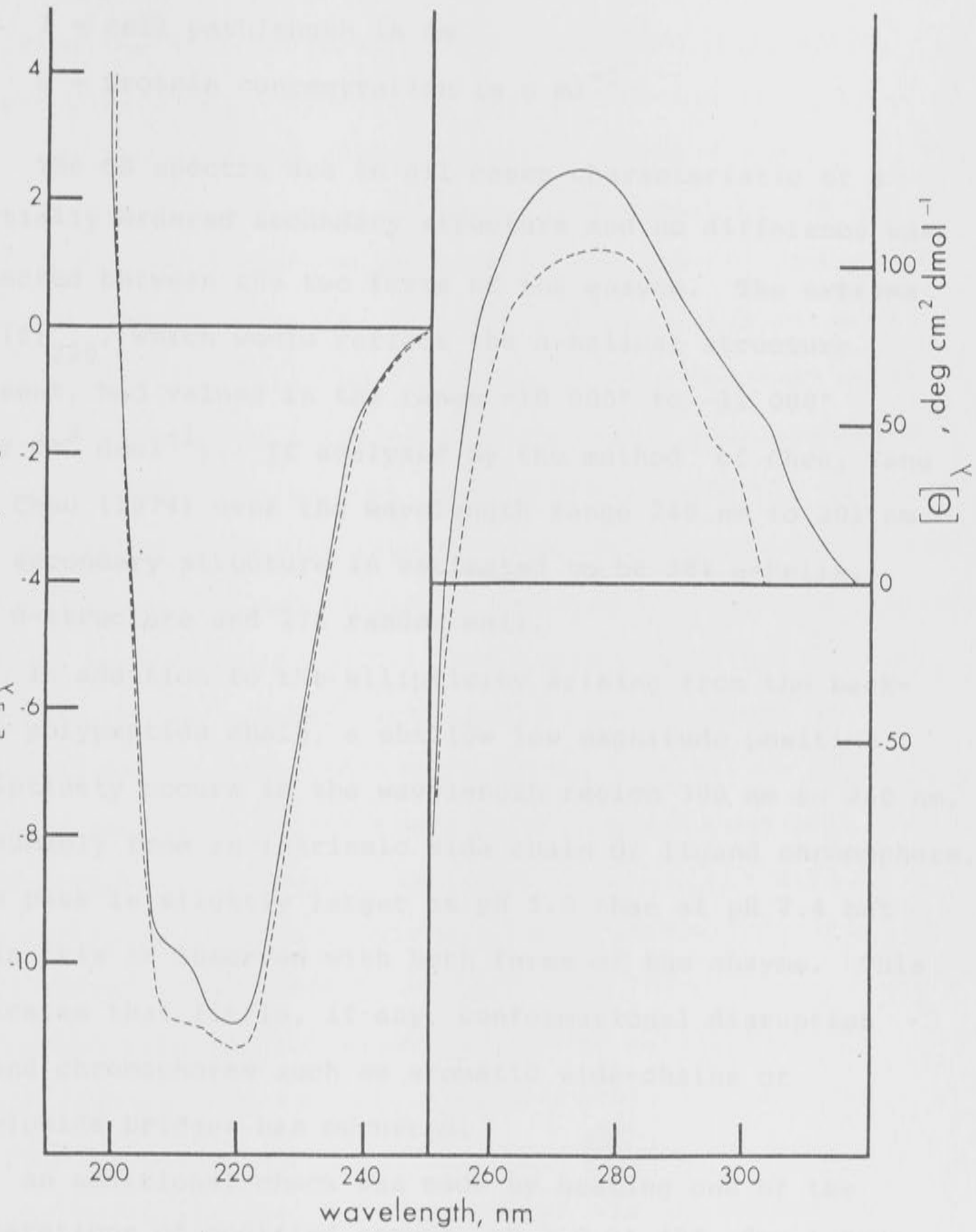


Figure 30 The aqueous circular dichroism spectra of substrate-modified sulphatase A (c=0.06%) in 5 mM Tris-HCl, pH 7.4, I=0.1, (———), and in 0.07 M sodium acetate buffer, pH 5.0, I=0.1 (----), at 10°C.

r = observed ellipticity in millidegrees

M_o = mean residue weight

l = cell pathlength in dm

c = protein concentration in g ml^{-1}

The CD spectra are in all cases characteristic of a partially ordered secondary structure and no difference was detected between the two forms of the enzyme. The extrema at $[\theta]_{220}$, which would reflect the α-helical structure present, had values in the range -10 000° to -11 000° (deg cm^2 $dmol^{-1}$). If analyzed by the method of Chen, Yang and Chau (1974) over the wavelength range 240 nm to 201 nm the secondary structure is estimated to be 38% α-helix, 35% β-structure and 27% random coil.

In addition to the ellipticity arising from the backbone polypeptide chain, a shallow low magnitude positive ellipticity occurs in the wavelength region 300 nm to 260 nm, presumably from an intrinsic side chain or ligand chromophore. This peak is slightly larger at pH 5.0 than at pH 7.4 but again this is observed with both forms of the enzyme. This indicates that little, if any, conformational disruption around chromophores such as aromatic side-chains or disulphide bridges has occurred.

An additional check was made by heating one of the preparations of modified enzyme, pH 5.0 at 45° for 1 hour to increase the proportion of native to modified enzyme. The initial velocity showed that the solution now contained 37% native enzyme, but, as was expected, no difference could be seen in the CD spectra.

These results differ from those found by Waheed and Van Etten (1980b) with the rabbit liver enzyme. They isolated the modified form of the enzyme and measured CD, UV and fluorescence spectra at both pH 7.5 and 4.5 of this solution and one containing only native enzyme. Differences were detected in the native enzyme at the two pH's in both the near ultraviolet CD and the fluorescence spectra. An increase in ellipticities at pH 4.5 and a quenching of the relative fluorescence were observed which were interpreted as tyrosine and tryptophan residues being buried during dimerization. The CD spectrum of the modified enzyme showed a 76% decrease in α-helical structure compared to the native enzyme. This was verified by the fluorescence spectra which showed a 67% loss of structure. The CD spectrum of the sulphate activated enzyme isolated in the same way as the modified enzyme showed that all of the protein secondary and tertiary structure had been lost. This preparation, however, had also lost all but 0.2% of its activity. As was observed, this evidence indicates that the activated enzyme they isolated had lost the structure required for sulphate-induced activation. It would appear, therefore, that denaturation had occurred during the isolation procedure. As the activity of the enzyme solutions used for the spectra was not recorded after the spectra had been taken it is difficult to determine whether the loss of structure is due only to inactivation or if it results from the instability of the enzyme and therefore a general denaturation occurring during isolation or the manipulations connected with taking

the spectra. It is noted in this regard that the CD and fluorescence spectra were taken at 37°C where the native rabbit liver enzyme is stable but the turnover-modified enzyme is readily denatured between 37°C and 52°C (Waheed and Van Etten, 1980b). The different spectra therefore demonstrate that in this case, either inactivation is directly responsible for a gross conformational change in the enzyme or causes changes which greatly alter its stability.

6.7 SUMMARY

It is thus clear that the substrate-induced modification of sulphatase A does not alter its molecular weight nor its self-association pattern. It also does not have a measurable effect on its conformation. The spectra obtained with the ox liver enzyme indicate that if any conformational change has occurred during modification it is small and within the experimental uncertainty of the methods used. It must be emphasized that with the large size of the molecule and the number of optically active residues present small changes in conformation which affect only a few residues or a small region in the molecule could easily be masked. For this reason the perturbation to the enzyme which might result from the binding of a sulphate molecule (see Chapter 7) may not be detected. The differences found with the rabbit liver enzyme reflect alterations in structure which are much more extensive than would be expected to result from the binding of two sulphate groups alone and suggest that with this enzyme other processes are involved in the inactivation.

7. ^{35}S-LABELLED SUBSTRATE-MODIFIED SULPHATASE A

7.1 INTRODUCTION

Although the inactivation of sulphatase A from many sources has been observed during the hydrolysis of aryl sulphates the nature of the change which this loss of activity reflected was not investigated fully. The formation of the inactive substrate-modified form of the enzyme occurs only during the hydrolysis of substrate and the inactivation observed under conditions such as standing in dilute solution or at low pH does not represent the same phenomena. In these cases activity is not increased with the addition of sulphate to the reaction mixture nor is there any evidence that the native form of the enzyme can be reformed. It is also interesting that in some cases it has been shown that the hydrolysis of the same substrates at other pH's does not lead to inactivation (Waheed and Van Etten, 1979). Thus, although the actual catalysis mechanism may be independent of the inactivation mechanism (Roy, 1978) the presence of substrate and the conditions of assay are important to the production of modified enzyme.

As the inactivation occurs only in the presence of substrate it would appear that an enzyme-substrate complex is formed at some point during the reaction. This raises the possibility that either the substrate or a reaction product remains bound to the modified enzyme. Such binding could either cause the inactivation or be a consequence of it. The existence of suicide substrates (Waley, 1980), or compounds which initially act as normal

substrates but then inactivate the enzyme by modifying an enzyme-substrate intermediate to leave the reaction uncompleted, is well known. This phenomena is distinguished from simple inhibition or inactivation by the fact that the inhibitor is acted upon by the enzyme as a normal substrate until an intermediate enzyme-substrate complex is prevented from reacting further. An example of this type of behaviour is the inactivation of the ß-lactamase I of *Bacillus cereus* by 6ß-bromopenicillanic acid. With this compound inactivation occurs after the normal acylation of the enzyme at Ser-70, when rearrangement of the penicilloyl-enzyme leads to the production of an abnormal state of the acyl group with which the enzyme can no longer function. This is thought to be due to the leaving group becoming irreversibly trapped by the enzyme when the 3-heteroazolidine ring of the 6ß-bromopenicillanic acid is opened (Loosemore *et al*, 1980; Cohen and Pratt, 1980). Another example of this type of behaviour is found with the inhibition of acetylcholinesterase by isopropyl methylphosphonofluoridate (Hovanec *et al*, 1977). The inhibition was shown to be accompanied by the formation of a covalent phosphorus-enzyme bond at a rate of 1×10^{7} M^{-1} min^{-1} at a serine residue present at the active site. The enzyme spontaneously reactivated and the return of activity was shown to parallel the displacement of this phosphonyl group. At pH 7.6 the reactivation proceeded with a $t_{\frac{1}{2}}$ of 154 minutes.

7.2 PRESENCE OF SUBSTRATE OR PHENOLIC PRODUCT

Early studies suggested that the modification of

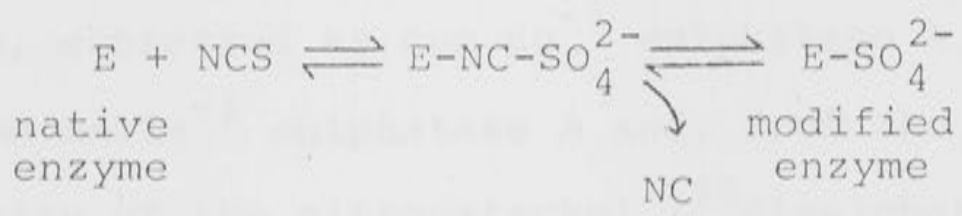
sulphatase A proceeded through an enzyme-substrate complex and for this reason it was thought that substrate may remain bound to the modified enzyme. Nicholls and Roy (1971) subsequently could find no spectroscopic evidence for the presence of either nitrocatechol sulphate or nitrocatechol in a preparation containing 50% modified sulphatase A.

This was also found with a sample of the modified enzyme used in these studies. The UV absorption of the solution at 280 nm at pH 7.4 was measured to determine the enzyme concentration and then the solution was made alkaline with NaOH. This treatment would be expected to release any loosely bound nitrophenol and shift the λ_{max} into the visible region. The absorbance at 280 nm at pH 7.4 was 1.715 giving a protein concentration of 2.45 mg ml^{-1} (23 μM). The presence of 1 mole of nitrocatechol per mole of enzyme would thus give an absorbance at 510 nm of 0.290 (assuming an ε_{510} = 12 600 identical to that of the free phenol) and similarly 1 mole of nitrocatechol sulphate per mole of enzyme would give an absorbance at 405 nm of 0.435 (assuming ε_{405} = 18 900) at alkaline pH. The absorbances detected at pH 11 were 0.019 at 510 nm and 0.059 at 405 nm indicating that less than 0.14 mole of either substance were present per mole of enzyme.

7.3 PRESENCE OF SULPHATE

The data from several other experiments would suggest that the sulphate moiety would be more likely to remain bound to the enzyme when it is modified, if in fact either of the products does. The experiments described in Chapter 4 on the effect of the two products of reaction on

the stability of the substrate-modified enzyme showed that nitrocatechol increased the rate at which the modified enzyme reverted to the native form while sulphate did not. This suggests the possibility that after hydrolysis the nitrocatechol is released but the sulphate remains associated with the modified enzyme. Incubating the modified enzyme with nitrocatechol could thus possibly shift the equilibrium between the native and modified forms either through removal of the sulphate or by allowing the normal release of the sulphate.



This proposal would be consistent with the fact that sulphate is a competitive inhibitor of the native enzyme and therefore the last released product of an uni-bi reaction. Also, data recently published by Roy (1978) indicates, on the basis of the kinetics of inactivation, that the formation of the modified enzyme proceeds through an enzyme-sulphate complex.

In order to determine if sulphate is in fact bound to the modified enzyme, native enzyme was inactivated with ^{35}S labelled nitrocatechol sulphate. The presence of substrate or of the sulphate moiety derived from the substrate could thus be detected in small amounts of enzyme. If radioactivity was detected in the modified enzyme preparation then the stoichiometry of binding could be easily calculated and the presence of the ^{35}S monitored

in experiments which alter the kinetic properties of the enzyme.

7.31 Method

Substrate-modified enzyme was prepared by the procedure described in Chapter 4 with the exception that nitrocatechol [^{35}S]sulphate was used as substrate. An aliquot of the incubation mixture was taken before the addition of enzyme to determine accurately the specific activity of the solution. After isolation the concentration and enzyme activity of the solution were measured as usual and two aliquots of different volume were counted. This value, expressed as cpm μg^{-1} sulphatase A, was converted to dpm $mmole^{-1}$ sulphatase A and, from the specific activity of the nitrocatechol [^{35}S]sulphate, to mmole SO_4^{2-} $mmole^{-1}$ sulphatase A. Finally this value was corrected to mmole SO_4^{2-} $mmole^{-1}$ modified enzyme from the kinetically determined amount of native enzyme remaining in the preparation.

To correct for any nonspecific adsorption of sulphate to the protein, native enzyme was incubated under the same conditions but having 20 mM $^{35}SO_4^{2-}$ present instead of substrate. This represents the approximate concentration of sulphate produced by the end of a 30 minute incubation under the conditions used to prepare the modified enzyme. The enzyme was then isolated as usual. In two preparations nonspecific adsorption was found to account for 0.16 mmole sulphate $mmole^{-1}$ enzyme. The ratios of bound sulphate to modified enzyme were subsequently corrected for these background counts.

7.32 Results

A. Substrate-Modified Enzyme

The results of five preparations of modified enzyme are given in Table 25. The data indicates that the modified enzyme has at some stage bound the sulphate moiety of the substrate molecule. A ratio of 1 mole of sulphate to 1 mole of modified enzyme was found suggesting that there is one active centre involved in the inactivation.

A series of experiments were done using the labelled enzyme to further study the relationship between the bound sulphate and the kinetics of the enzyme and also as a check that the bound sulphate is directly related to the inactivation. The enzyme was treated in several ways which were known to affect its activity and the amount of radioactivity was determined after each. These results are also given in Table 25. It can unequivocally be stated only that the ^{35}S is bound as there is no direct evidence that it remains in a sulphate group. Alteration of the sulphate would not be expected, however, under the conditions used.

B. Reversion to Native Enzyme

A sample of one preparation of the labelled modified enzyme which had been isolated and characterized, was heated for 5 hours at 37°C in 0.05 M Tris-HCl pH 7.4, 0.5 M NaCl. The solution was then applied to a Sephadex G-25 column (1.5 × 13 cm) and eluted with 0.01 M Tris-HCl pH 7.4, 0.15 M NaCl in order to remove any sulphate which may have dissociated from the enzyme. One ml fractions were collected and the enzyme peak determined from the absorbance at

TABLE 25

^{35}S-LABELLED SUBSTRATE-MODIFIED SULPHATASE A

Prep	Activity of $^{35}SO_4^{2-}$ dpm µmole^{-1}	Protein Concentration mg ml^{-1}	% Inactivation	$\frac{^{35}S}{Enzyme}$	$\frac{^{35}S}{modified\text{-}enzyme}$	Corrected
1	117 500	0.65	0.84	0.95	1.13	0.94
2	76 000	0.64	0.87	1.15	1.32	1.14
3	95 400	0.39	0.91	1.31	1.44	1.26
4	92 600	0.17	0.94	0.89	0.95	0.78
5	314 300	0.61	0.92	1.12	1.22	1.06

Treatment	Protein Concentration mg ml^{-1}	% Inactivation	$\frac{^{35}S}{modified\ enzyme}$
SO_4^{2-} activation	0.07	0.61	1.10
	0.05	0.77	1.54
Incubation 37°C for 5 h	0.65	0.57	0.99
	0.39	0.52	1.07
		before dialysis / after dialysis	
Dialysis against SO_4^{2-}	0.65	0.84 / 0.83	0.0
	0.39	0.91 / 0.86	0.0

280 nm. The peak fractions were pooled, the concentration of enzyme determined from its absorbance, the enzyme activity by pH-stat assay and aliquots were counted. As shown in Table 25 approximately half of the modified enzyme reverted to its native form as determined kinetically. This would be predicted from the $t_{\frac{1}{2}}$ of 5 hours at 37°C and is consistent with the values obtained previously with unlabelled enzyme. The important observation is that the ratio of sulphate to modified enzyme remains 1:1. Thus the sulphate is not irreversibly bound and its release corresponds to reversion of the modified to the native form of the enzyme. This provides further evidence that the enzyme activity regained on heating represents a reforming of the native state of the enzyme and is different from that observed with the addition of an activator.

C. Activation

A sample of the ^{35}S-labelled modified enzyme was activated in the pH-stat as before using unlabelled nitrocatechol sulphate as substrate and unlabelled K_2SO_4 as activator. After 60 minutes the enzyme was isolated and characterized as usual. Again, although substantial reversion to native enzyme was observed, the ratio of sulphate to modified enzyme was approximately 1:1. The activation mechanism therefore does not involve removal of the bound sulphate. This further clarifies what was shown by comparing the kinetic curves of the native, modified and activated enzymes (section 4.23). It was noted from this data that the activation did not produce a

third stable form of enzyme and that the enzyme isolated after sulphate activation had the characteristics of the modified enzyme. The fact that the ^{35}S is retained by the enzyme through the activation in a quantity consistent with the 1:1 ratio demonstrates that it is the modified enzyme which is present. It further indicates that the sulphate added as activator does not displace the sulphate bound during inactivation and eliminates the possibility that the modified enzyme may have been reformed during the reaction or the isolation procedure. In this case the sulphate bound would not contain ^{35}S. The activating sulphate is therefore increasing the activity of the modified enzyme by some means which does not require removal of the bound sulphate. The nature of this mechanism remains unknown.

7.4 NATURE OF BOUND SULPHATE

The interaction between the sulphate group and the sulphatase A molecule is clearly strong enough to withstand chromatography on Sephadex G-25 and dialysis against buffers of pH 7.4 and pH 5.0 with I=0.1. This suggests that a covalent bond has been formed and Waheed and Van Etten (1980b), who demonstrated that the substrate-modified rabbit liver sulphatase A contained two moles of ^{35}S per mole of enzyme monomer, believe this to be the case and refer to the process as "covalent modification". Other evidence, however, obtained with the ox liver enzyme suggests that the bond cannot be covalent and different forms of interaction must be considered. This is discussed below.

It is probable that the sulphate is bound at or near to the active site as it originates from the hydrolysis of a substrate molecule. This could be determined if an active site titrant or label were known for sulphatase A. The only instance of such a compound being found was reported by Benkovic and Fedor (1972) who found o-nitrophenyl oxalate to be a suitable active site titrant for an arylsulphatase from *Aspergillus oryzae*. Extrapolation to complete inhibition of a plot of residual enzyme activity versus inhibitor concentration showed the reaction of two moles of inhibitor per mole of sulphatase. This compound is difficult to prepare (Roy, private communication) and has not been obtained in this laboratory. Even if it were available this approach would not be useful under these conditions as the modified enzyme has no activity. A reagent which would form a covalent bond at the active site is required but has not yet been found for sulphatase A. If such a compound were detectable itself, for example by a characteristic absorbance or fluorescence peak, or if the enzyme derivative showed altered spectroscopic properties such that the concentration of reagent could be accurately determined, then the number of active sites per molecule of enzyme could be calculated. A value for the modified enzyme less than that for the native enzyme would suggest that the $^{35}SO_4^{2-}$ was bound to the active site and preventing the interaction with the titrant. However, the hypothesis that an extra binding site is exposed on the modified enzyme may make interpretation of such data difficult.

Much work has been done to determine which amino acids are essential to the catalytic activity of sulphatase A using general protein reagents which are specific for a functional group or amino acid. This allows some suggestions to be made regarding which amino acids may be involved in the interaction with the sulphate group. The evidence obtained for different amino acid residues is summarized below.

The one common residue required for activity in all sulphatases A tested is histidine. Jerfy and Roy (1974) showed this to be the case with the ox liver enzyme by treating it with diazonium salts. Lee and Van Etten (1975b) came to the same conclusion using the rabbit liver sulphatase A from the pH dependence of inactivation by diethyl pyrocarbonate which indicated a pK of 6.5-7.0 for the reacting group. Maengwyn-Davies and Friedenwald (1954) also obtained evidence consistent with the presence of an essential histidyl residue in the rabbit liver enzyme using diazotized sulphanilic acid although this was not the only possible interpretation of the data. Similarly histidine was shown to be a possible essential residue for the catalytic activity of the enzyme from human placenta (Gniot-Szulzycka, 1974) and ox brain (Bleszynski and Leznicki, 1967).

With the ox liver sulphatase A tyrosyl residues may also be required for activity as the enzyme is inactivated by N-acetyl-imidazole or tetranitromethane (Jerfy and Roy, 1969). The results with the latter are ambiguous, however, because of the many side reactions, such as the oxidation

of sulfhydryl groups and methionine, and modification of tryptophan and histidine, observed when proteins are treated with this reagent. Neither SH nor amino groups appear to be required by the ox liver enzyme for activity. Thiols were demonstrated not to be a reactive group in rabbit liver enzyme by Maengwyn-Davies and Friedenwald (1954). James has shown that the human liver sulphatase A is inactivated by treatment with arginine-specific reagents (1979) although no inhibition of the ox liver enzyme was found after this treatment (Roy, private communication).

It is possible that the sulphate has formed a sulphate ester with tyrosine or a sulphamate with the ring nitrogen of histidine, both being residues which are probably essential for the activity of the native enzyme. It is also possible for sulphate to react with a serine, threonine or tryptophan residue to form a sulphate ester, an amino group to form a sulphamate, or a cysteine residue to produce a thiosulphate. Many of these sulphate esters are stable and occur naturally. Tyrosine O-sulphate is known to be present in peptides such as fibrinogen and fibrin (Jevons, 1963) and is excreted in human urine (Tallan *et al*, 1955). Thiosulphates also occur naturally and participate in the interconversions of sulphate and sulphite. Serine O-sulphate has been found to be accumulated by brown adipose tissue (Powell *et al*, 1967) although the reason for this is unknown. Imidazole sulphates have not been observed naturally but have been synthesized (Mayers and Kaiser, 1968) and shown to be stable. It was suggested that they may play a role in sulphate transfer in biological systems.

It is interesting to note that if the sulphate is in fact bound to the active site of the native enzyme, rendering it inactive, then the activity of the modified enzyme would need to be associated with a separate active centre.

If in fact the sulphate is bound covalently it should be possible to determine the residue to which it is bound by doing a peptide map of the labelled enzyme and determining which amino acids are present in the peptide containing the ^{35}S. Three different digestion methods were tried but in each case the radioactivity indicated only free inorganic sulphate was present after electrophoresis. The bond was therefore not sufficiently stable to remain intact through the digestions and subsequent treatment.

The labelled enzyme was digested in 5% trypsin at 5°C overnight, lyophilized, redissolved and precipitated with glacial acetic acid. As there was very little precipitate a chymotrypsin digestion was not done and the solution was again lyophilized and the solid taken up in 50 µl of solution. A second sample was treated with 40% subtilisin A overnight and a third with urea and subtilisin overnight. The samples were applied to paper and separated by electrophoresis for half an hour along with an aliquot of $^{35}SO_4^{2-}$. After drying the strips were cut into 1 cm sections and counted in scintillation fluid. The mobility of the radioactivity in all cases corresponded to that of the free sulphate.

Further attempts were made to identify the nature of the sulphate-enzyme interaction. It was found that denaturing the enzyme with 4.5 M urea, 1% SDS or 30% ethanol all

led to the loss of the sulphate suggesting that the binding relies heavily on the conformation of the protein. This would explain the difficulty encountered in the peptide mapping.

A sample of the labelled enzyme was dialyzed against a solution of 10 mM K_2SO_4 in 0.1 M Tris-HCl pH 7.5 for 3 days and then against the buffer alone for 3 days. As is shown in Table 26, after this time the enzyme activity was found to have remained essentially constant although the radioactivity associated with the enzyme was lost. As the information given above indicates that if the sulphate group is lost the enzyme returns to its native form, this data suggests that there has been an exchange between the enzyme-bound sulphate and the sulphate in solution.

TABLE 26

DIALYSIS OF ^{35}S-LABELLED SUBSTRATE-MODIFIED SULPHATASE A AGAINST K_2SO_4

	v_o μmoles min^{-1} mg^{-1}	counts
I.		
before dialysis	26	123 dpm
after dialysis	28	0
II.		
before dialysis	33	114
after dialysis	33	0

Similarly when the unlabelled modified enzyme was dialyzed against 10 mM $^{35}SO_4^{2-}$ in 0.1 M Tris-HCl pH 7.5

for 3 days and then exhaustively against the buffer alone to remove unbound $^{35}SO_4^{2-}$, the enzyme became labelled. An aliquot of the original $^{35}SO_4^{2-}$ solution, which had been made up from 2 mCi ml^{-1} $^{35}SO_4^{2-}$ in aqueous solution (Radiochemical Amersham) diluted with K_2SO_4 to give a concentration of 10 mM, was counted to determine the specific activity of the sulphate solution. This value (200 dpm $nmole^{-1}$) was then used to calculate the ratio of sulphate to enzyme as before. It was found that in two experiments 76% and 84% of the sulphate had exchanged.

TABLE 27

DIALYSIS OF SUBSTRATE-MODIFIED SULPHATASE A AGAINST $^{35}SO_4^{2-}$

	v_o µmoles $min^{-1}mg^{-1}$	counts dpm $nmole^{-1}$	Activity $^{35}SO_4$ dpm $nmol^{-1}$	% Exchange
I.				
before dialysis	18	0		
after dialysis	16	141	200	76%
II.				
before dialysis	13	0		
after dialysis	14	157	200	84%

Similar experiments were then done to test the susceptibility of the sulphate group in several compounds to exchange with free sulphate. The materials tested are

listed in Table 28, along with the exchange observed after standing for 4 days at 5°C in a 10 mM $^{35}SO_4^{2-}$ solution. As can be seen very limited exchange has occurred, being less than 1% in all cases except ß-naphthyl sulphate. Approximately 0.1 nmole of each compound was dissolved in 2 ml of 0.1 M Tris-HCl pH 7.5. These solutions were then made 10 mM $^{35}SO_4^{2-}$ and a zero time control obtained by immediately removing 0.5 ml, precipitating the inorganic sulphate with a slight excess of $BaCl_2$ (8 µmoles for 0.5 ml), centrifuging and then counting the supernatant. After 4 days the remaining solutions were treated in the same way and the amount of sulphate exchange calculated. These experiments therefore do not give any indication as to the type of bond which may be present but do emphasize the lability of the interaction. It is apparently stabilized by the protein environment surrounding it, perhaps by the close proximity of other charges, to the extent that it will remain associated with that portion of protein as long as there is no unfolding or other ions present which can displace it and be retained with equal ease.

It is possible that the sulphate-enzyme bond is more ionic in character than covalent and may be more aptly described as the SO_4^{2-} being trapped in the protein structure. This could result from a conformational change in the enzyme, associated with the substrate-induced inactivation, altering the environment around the SO_4^{2-} in such a way that it is not released. This "trapping" of the SO_4^{2-} could be caused by conformational movement which provided steric

hindrance to SO_4^{2-} release or which altered the charge structure of the enzyme in that region such that the SO_4^{2-} was held. This would possibly explain why exchange can take place, why denaturing the enzyme releases the SO_4^{2-}, and why the substrate-modified enzyme can easily revert to its native form.

TABLE 28

SULPHATE EXCHANGE IN SULPHATE ESTERS

Compound	$\frac{\text{cpm}}{\mu\text{mole ester}}$	% exchange
p-nitrophenyl sulphate	17	0.01
β-naphthyl sulphate K-salt 4-H_2O	2,233	1.5
potassium β-naphthyl thiosulphate	53	0.04
potassium 4-pyridone 1-sulphonate	59	0.04
potassium β-naphthyl sulphamate	128	0.08
tyrosine-o-sulphate K-salt	34	0.02

7.5 SUMMARY

This work with radioactive substrate has allowed more conclusive information to be obtained than kinetic data alone has in the past. It has shown that the sulphate moiety derived from the substrate is associated with the modified enzyme and remains so as long as the enzyme remains modified. Reactivation was demonstrated not to involve removal of this sulphate nor formation of a third stable form of the enzyme. It appears that loss of this bound

sulphate parallels the reversion to native enzyme and that whether the failure to release the sulphate product during catalysis causes inactivation or is a consequence of it the sulphate remains bound as long as the enzyme is modified.

Waheed and Van Etten (1980b) have recently published data for sulphate binding to rabbit liver sulphatase A during inactivation. They found a 2:1 ratio of substrate derived sulphate to enzyme in their isolated enzyme preparation but did not study the labelled enzyme further. In this case also nitrocatechol [^{35}S]sulphate was used as substrate but Ba^{2+} was present in the inactivation mixture which proves that the sulphate has not been released by the enzyme at any stage. The mechanism of inactivation therefore appears to involve binding the sulphate group of the substrate with sulphatase A from at least two different sources.

The inactivation is similar to that found with β-lactamase I and acetylcholinesterase but with some important differences. Unlike β-lactamase I, the formation of the inactive enzyme is readily reversible and unlike both of these other enzymes, with sulphatase A catalytic activity can be induced in the inactive enzyme. The behaviour of β-lactamase I with quinicillin as substrate is perhaps more comparable as in this case the inactivation is completely reversed once all of the quinicillin is hydrolysed. Kinetic evidence suggests that it is caused by the formation of a second form of the enzyme which is conformationally different and will not hydrolyse benzlpenicillin only quinicillin and that at a much lower rate. Again it is

thought that the quinicillin conjugate arises from a state of the enzyme-substrate complex present during the normal catalytic cycle (Virden *et al*, 1978).

8. DISCUSSION

The exact nature of the physical changes which are reflected in the anomalous kinetics remain unknown although some stages of the reaction have been clarified by recent work. Baum and Dodgson (1958) originally suggested several possible explanations for the unusual time course of the reaction but favoured one which involved the exposure of a second binding site on the enzyme through interaction with substrate. The possibility that there were two separate arylsulphatases present, one having a greater activity but being labile and rapidly inactivated in the presence of substrate and a second being more stable but requiring the presence of an anion, such as sulphate, for activity, was thought unlikely. This hypothesis does not explain why a linear progress curve was observed when $P_2O_7^{4-}$ was present in the reaction mixture nor the interconvertibility of the two enzymes. Another possibility was that the enzyme was being sulphated in a manner analogous to the acylation of chymotrypsin during hydrolysis of p-nitrophenyl acetate. This hypothesis was rejected on the basis of the stoichiometry of the inactivation reaction because approximately 200 substrate molecules would be hydrolysed per molecule of enzyme before inactivation was complete, and also because it did not seem likely that sulphate would activate an enzyme-sulphate complex. The former is an objection only if it is assumed that the inactivation mechanism is inseparable from the catalytic reaction and therefore that inactivation must occur each time a substrate molecule is hydrolysed. Much

evidence now exists to indicate that this is not the case and that sulphation of the enzyme is associated with the substrate-induced inactivation. Baum and Dodgson themselves point out that the two reactions respond differently to changes in pH and temperature which suggests the inactivation does not stem from a mandatory step of the catalytic mechanism. Optimum conditions for the formation of the inactive, modified enzyme may therefore be related to the optimum conditions for hydrolysis but not necessarily identical to them. The kinetic study of the inactivation of the ox liver enzyme (Roy, 1978) further illustrates this point. It was shown in this investigation that k, a rate constant for inactivation obtained through extrapolation of measured values to infinite substrate concentration, was constant over a range of different substrates which gave different values for V_o with the native enzyme. This suggests that the rate of inactivation is independent of the rate of hydrolysis. It was further shown that theoretically the inactivation reaction could proceed in the absence of the hydrolytic reaction as k*, the apparent velocity constant for inactivation, was finite when v_o was zero. The stoichiometry of this reaction need not, therefore, be an objection if the mechanism is considered to be slightly different from that found with chymotrypsin in that the inactivation is not a necessary consequence of the binding of substrate. Experiments using radioactively labelled substrate have shown that sulphation of the enzyme is in fact associated with the substrate-induced inactivation. With the ox liver enzyme one mole of

sulphate is bound per mole of modified enzyme (Prosser and Roy, 1980) and with the rabbit liver enzyme the ratio is two to one (Waheed and Van Etten, 1980b). It has still not been determined, however, how this sulphate is bound to the enzyme and whether its binding causes the inactivation or is a consequence of it. As the bond is very labile it seems possible that conformational movement in the enzyme-sulphate complex has occurred which traps the sulphate group. If the sulphate were being held within the enzyme molecule in this way rather than being covalently bound to one of the amino acid residues its loss on treatment with denaturing reagents such as urea and SDS would be easily explained.

The reaction mechanism which Baum and Dodgson (1958) proposed and Nicholls and Roy (1971) discussed is consistent with the observations made with the ox liver sulphatase A. Figure 3 is a diagrammatic representation of this mechanism. In their scheme a new binding site is slowly exposed on the enzyme through its interaction with substrate to form the substrate-modified enzyme. This form of the enzyme may be produced either during the catalytic reaction or from an enzyme-substrate complex without the release of products. The second site can bind substrate to produce an inactive complex or with the products of the reaction or inhibitors of the initial velocity to produce an active complex. If these latter compounds are present in excess, they also bind to the active centre (the original binding site) and inhibit the reaction. Baum and Dodgson proposed that the second binding site, exposed during modification, was purely regulatory and therefore that the

enzyme complex having substrate bound to this site and sulphate to the original binding site was also inactive. It was not suggested by either of these authors whether the FS complex was active or not but both postulate that the inactive enzyme produced under normal assay conditions is an FS_2 complex.

This general pattern seems to be correct in that the modified enzyme appears to have two binding sites and that both substrate and an activating anion must be bound for the enzyme to be active. When only the modified form of the enzyme is present the rate of reaction is therefore governed by competition between the substrate and reaction products for the binding sites. As before, the evidence for this comes from the substrate and sulphate inhibition observed if their concentrations are raised above optimum levels and from the fact that neither substrate nor sulphate alone can induce inactivation in the modified enzyme. The hypothesis that the second binding site is not catalytically active may not be correct however. It has been demonstrated that the sulphate moiety derived from the substrate is associated with the modified enzyme and because this sulphate is produced by the hydrolytic reaction it has been assumed to bind at or very near to the active site. This raises the possibility that the bound sulphate may block the active site and thereby prevent further catalysis at that centre. The second binding site may therefore be the catalytic site for the modified enzyme. It was shown, using the modified enzyme inactivated with nitrocatechol [^{35}S]sulphate, that activation of this enzyme occurred

without the loss of the inactivating sulphate. Activation therefore, would appear not to require the unblocking of the original active site. This could mean either that the sulphate is not bound to the active site, is not bound to an essential amino acid residue, or does not provide sufficient steric hindrance to inhibit the reaction; or that a second active site is available. The only answer to this question seems to lie in finding a compound which will irreversibly bind to the original active site without preventing the formation of the modified enzyme. If activity could be restored after reaction with such a compound then a second active site must be present.

If the modified enzyme is considered to be an enzyme-sulphate complex the scheme described above seems to be consistent with the experimental observations. This complex appears to be capable of binding both sulphate and substrate to be activated when the relationship between the concentration of each is correct and to be inhibited if the binding of one or the other is dominant. It is not known whether the activating sulphate is released from the active FSI complex after the hydrolysis of the substrate to reform the inactive enzyme or whether it remains bound and therefore the enzyme active. As the activated enzyme could not be isolated and release of the sulphate along with the reaction products is consistent with the formation of a steady state it is thought that the inactive enzyme is reformed at the end of the catalytic reaction. The inactive enzyme being the enzyme-sulphate complex formed during the modification reaction.

The mechanism behind the activation of the substrate-modified emzyme remains a mystery. The kinetics of the reaction suggest that there is a slow isomerization of the inactive modified enzyme-substrate-sulphate complex to an active form which then hydrolyses the substrate. Although no evidence exists to prove the difference between the inactive and active modified forms is conformational the data available to date suggests that this is the most likely explanation. The binding of the activator alone is not enough to activate the modified enzyme as is demonstrated by the unaltered time course observed after preincubation with sulphate. Apparently the presence of both activator and substrate is required before activation will begin. Nicholls and Roy (1971) suggested that the slow rate of activation was due to the slow release of a substrate molecule from FS_2 to give FS which could then bind the activator to produce the active complex FSI. If this is the case, the slow rate of activation observed after preincubation with sulphate would similarly be explained by a slow reaction $FI_2 \rightarrow FI$ which would then bind substrate. It does not explain why the rate of activation is the same when both compounds are present from the beginning of the reaction and therefore a step after the binding of both ligands appears to be rate limiting. It is possible that a slow polymerization reaction is involved but this does not seem likely as there is no evidence to suggest different polymeric forms have different kinetic properties or that the enzyme would associate under these conditions. A conformational change in the enzyme molecule seems a more probable explanation.

The increase in the rate of reversion of the modified enzyme to the native enzyme observed in the presence of sulphate and substrate also bears mentioning. It has been demonstrated by Nicholls and Roy (1971) and in Chapter 4 of the present work that sulphate alone does not affect the rate at which the substrate-modified enzyme converts back to the native enzyme when heated in buffer. The addition of substrate alone increases the fraction of modified enzyme present probably as a result of continued inactivation. If both compounds are present, however, the reversion is facilitated. It should be emphasized that this reaction is not equivalent to the activation reaction as the concentration of native enzyme found after 30 minutes of activation could not account for the activity observed. The increase in the rate of reversion may reflect changes in the activated modified enzyme which increase the lability of the enzyme-sulphate interaction. For instance, if a conformational change is responsible for the sulphate not being released and therefore the modification, and a second conformational change is associated with the activation, it may be that the active conformation more closely resembles that of the native enzyme and therefore the sulphate is less tightly held. It may also be that if the active site in the activated modified enzyme is the original active site, the slight conformational motions which would be associated with the hydrolysis and release of products may be sufficient to increase the probability that the sulphate is released. This assumes that the sulphate is bound at or near to this active site and that

it is "held" by the protein conformation rather than being covalently bound.

The anomalous kinetics of sulphatase A are unique in that strictly analogous behaviour has not been observed with any other enzymes. The inactivation of many enzymes during catalysis has been noted and these mechanisms may be comparable to the sulphatase A reaction to a limited extent but differences are evident. As mentioned before the sulphation of the modified enzyme may be similar to the acylation of α-chymotrypsin and the irreversible binding of benzylpenicillanic acid to β-lactamase I but neither of these inactivated enzymes are activated by the products of reaction. Indeed the situation where anions which inhibit the initial stages of the reaction, i.e. the initial velocity and the substrate-induced inactivation, and activate a second form of the enzyme seems to be unique to sulphatase A. In the case of β-lactamase I acting with quinicillin as substrate a second form of the enzyme is produced which will slowly hydrolyse quinicillin but not penicillin so that an inactivation of the penicillinase activity is observed. As with the inactivation of sulphatase A a conformational change is thought to play a role in the formation of the second form of enzyme. In this respect and also in the reversibility of the reaction, β-lactamase I appears to most closely resemble sulphatase A. With β-lactamase I the penicillinase activity is fully restored once the quinicillin has been fully hydrolysed but again penicillinase activity has not been shown to be induced in the quinicillin-modified enzyme. The mechanism

behind the anomalous behaviour of sulphatase A is therefore of interest kinetically if not physiologically.

The physiological relevance, if any, of the inactivation is obscure. If cerebroside sulphate is the only physiological substrate for this enzyme then it is probably of no importance at all as it does not occur during the hydrolysis of this substrate. Knowledge of the mechanism behind the anomalous kinetics and of the factors contributing to the apparently different mechanism followed with these two substrates would however, help to define the action of the enzyme and its control. For instance, it is still not clear why bile salts or an activator protein are required for cerebroside sulphatase activity and what role, if any, the hydrocarbon portion of the sulphatide has in preventing inactivation. It seems very unlikely, however, that cerebroside sulphate is the only physiologically important substrate for this enzyme and the possibility that the modification reaction is active to some extent *in vivo* when other compounds are hydrolysed cannot yet be dismissed as it is observed experimentally with other substrates of possible physiological significance. It is unfortunate, in this regard, that the kinetic details of the hydrolysis of these compounds has been ignored. As mentioned in the introduction, an appreciable amount of the work related to arylsulphatases is directed towards defining their role *in vivo*. To do this the activity of these enzymes has been tested against many naturally occurring sulphate esters where only a positive or negative response is

required. The details of the kinetic behaviour were not of interest to these investigators and therefore were not studied. The information potentially available from studying the kinetics of sulphatase A with these other naturally occurring substrates and with synthetic substrates designed to elucidate the importance of specific structural parts of the molecule to its hydrolysis and the mode of action of the enzyme, could help to define the modification reaction.

Many questions concerning the mechanism of the substrate-induced inactivation of sulphatase A when acting as an arylsulphatase remain unanswered. Direct evidence for the presence of two binding sites in the modified enzyme has not been obtained. Use of an active site label seems to be the only method of determining the number of binding sites which can be successfully applied in this situation and synthesis of such a label has not been accomplished. Although it has been shown that during modification the sulphate derived from the substrate becomes associated with the modified enzyme, the nature of the enzyme-sulphate bond is not clear, if indeed it is an actual bond. It is also not known whether the formation of this bond causes the inactivation or is a consequence of it and subsequently is unimportant to the kinetic properties of the modified enzyme. The catalytic activity of the FS complex is undetermined and it does not seem possible to answer this question on the basis of kinetic data alone. The number of substrate molecules bound to the enzyme must be known as well as the rate at which

products are formed. Determination of the equilibrium constants for the reactions $F + S \rightarrow FS$ and $FS + S \rightarrow FS_2$ may help to solve this problem.

Although it seems improbable, the possibility that the ^{35}S bound to the substrate-modified enzyme is actually in a nitrocatechol sulphate molecule has not been conclusively proven. The spectroscopic evidence that there was no nitrophenol present in the preparation of the modified enzyme was based on the assumption that the extinction coefficient of the bound nitrophenol would be similar to the one measured in free solution. It is possible that the absorbance spectra is altered in the protein environment and therefore that the phenol was not detected. This point should be easily resolved by the synthesis of a substrate labelled with ^{14}C.

APPENDIX 1

The equilibria shown below describe the binding of two different ligands (B and C) to an enzyme (E) which has two binding sites. The system is simplified by assuming that the binding of the second ligand is not influenced by which ligand is already bound and therefore, the equilibrium constants for the reactions EB + B and EC + B are said to be equal, as are those for the reactions EB + C and EC + C.

$$\underset{(e\text{-}e_1\text{-}e_2\text{-}e_3\text{-}e_4\text{-}e_5)}{E} + \underset{(b)}{B} \overset{K_1}{\rightleftharpoons} \underset{(e_1)}{EB}$$

$$\underset{(e\text{-}e_1\text{-}e_2\text{-}e_3\text{-}e_4\text{-}e_5)}{E} + \underset{(c)}{C} \overset{K_2}{\rightleftharpoons} \underset{(e_2)}{EC}$$

$$\underset{(e_1)}{EB} + \underset{(b)}{B} \overset{K_3}{\rightleftharpoons} \underset{(e_3)}{EB_2}$$

$$\underset{(e_2)}{EC} + \underset{(c)}{C} \overset{K_4}{\rightleftharpoons} \underset{(e_4)}{EC_2}$$

$$\underset{(e_1)}{EB} + \underset{(c)}{C} \overset{K_4}{\rightleftharpoons} \underset{(e_5)}{EBC}$$

$$\underset{(e_2)}{EC} + \underset{(b)}{B} \overset{K_3}{\rightleftharpoons} \underset{(e_5)}{EBS}$$

The concentration of each enzyme complex in the above system can be written as follows, assuming $b \gg e$ and $c \gg e$ and therefore treating the K's as Michaelis constants.

$$e_1 = \frac{(e-e_1-e_2-e_3-e_4-e_5)\cdot b}{K_1} \tag{1}$$

$$e_2 = \frac{(e-e_1-e_2-e_3-e_4-e_5)\cdot c}{K_2} \tag{2}$$

$$e_3 = \frac{e_1\cdot b}{K_3} \tag{3}$$

$$e_4 = \frac{e_2\cdot c}{K_4} \tag{4}$$

$$e_5 = \frac{e_1\cdot c}{K_4} = \frac{e_2\cdot b}{K_3} \tag{5}$$

By substituting equations (2)-(5) into equation (1) the concentration of e_1 can be defined in terms of e,b,c, and the equilibrium constants. Similarly expressions for e_2, e_3, e_4 and e_5 can be derived.

$$K_1e_1 = (e-e_1-e_2-e_3-e_4-e_5)b$$

$$= \left(e-e_1 - \frac{K_3e_1c}{K_4b} - \frac{e_1b}{K_3} - \frac{K_3e_1c^2}{K_4^2b} - \frac{a_1c}{K_4}\right)b$$

$$e_1 = \frac{K_3K_4^2eb^2}{K_1 + K_3K_4^2b^2 + K_3^2K_4bc + K_4^2b^3 + K_3^2bc^2 + K_3K_4b^2c}$$

$$= \frac{K_3K_4^2eb^2}{X} \tag{6}$$

$$e_2 = \frac{K_3^2 K_4 ebc^2}{X} \quad (7)$$

$$e_3 = \frac{K_4^2 eb^3}{X} \quad (8)$$

$$e_4 = \frac{K_3^2 ebc^2}{X} \quad (9)$$

$$e_5 = \frac{K_3 K_4 eb^2 c}{X} \quad (10)$$

A plot of the concentration of the enzyme complex EBC (e_5) as a function of the concentration of B, at various values of C is shown in Figure 31. If it is assumed that substrate-modified sulphatase A has two binding sites and that the activity observed after the addition of sulphate is due primarily to an enzyme-substrate-sulphate complex, then the activation can be compared to this simple model and the concentration of EBC should be related to the observed velocity. The curves shown in the figure were calculated from equation (10) with $K_1 = K_2 = 1$ and $K_3 = K_4 = 0.5$. These values were chosen because K_m for nitrocatechol sulphate is 1 mM for native sulphatase A and the equilibrium constant for the reaction E(NCS) + (NCS) → $E(NCS)_2$, which would be equivalent to K_3, was estimated to be 0.5 by Nicholls and Roy (1971). It is noted, however, that no units are given for this value. Values for K_2 and K_4, representing the binding of sulphate, have arbitrarily been set equal to K_1 and K_3 as experimental data suggests that they are similar to those for nitrocatechol sulphate binding.

A similar pattern emerges with both the plot of the observed activated velocity versus substrate concentration at various sulphate concentrations (Figure 14) and the plot of the concentration of EBC versus B at various concentrations of C (Figure 31). The hypothesis that the shape of the curves in Figure 14 resulted from a restriction on the maximum velocity observed in each case due to the formation of FS_2 and FI_2 is therefore consistent with this simple model. The calculated curves are of course very sensitive to the K values chosen. For example, if K_3 and K_4 are raised to 0.75 while K_1 and K_2 are kept at 1, the maxima are shifted to lower concentrations of B and the curves decrease more rapidly once the maximum has been attained. Because accurate values for these equilibrium constants are not known, this model serves only to demonstrate that the experimental observations can be qualitatively explained in this way.

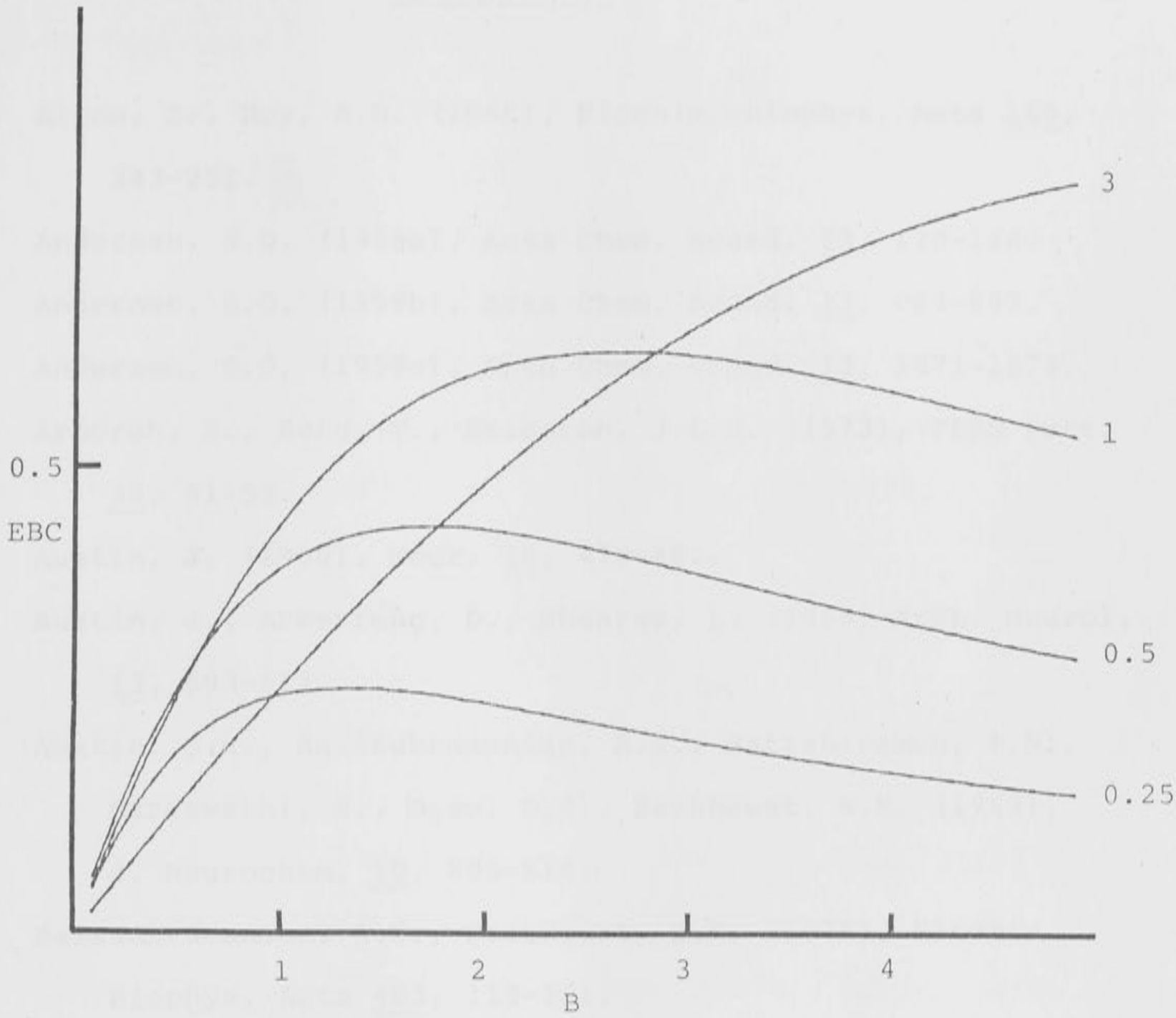


Figure 31 The concentration of EBC calculated from equation (10), given in the text, with e = 1, $K_1 = K_2 = 1$ and $K_3 = K_4 = 0.5$. The value of b was varied from 0.01 to 5 and c was set to 0.25, 0.5, 1.0 and 3.0 as shown at the right of the curves.

BIBLIOGRAPHY

Allen, E., Roy, A.B. (1968), Biochim. Biophys. Acta 168, 243-251.

Andersen, S.O. (1959a), Acta Chem. Scand. 13, 120-126.

Andersen, S.O. (1959b), Acta Chem. Scand. 13, 884-887.

Andersen, S.O. (1959c), Acta Chem. Scand. 13, 1671-1679.

Arborgh, B., Berg, T., Ericsson, J.L.E. (1973), FEBS Lett. 35, 51-53.

Austin, J. (1960), Neur. 10, 470-483.

Austin, J., Armstrong, D., Shearer, L. (1965) Arch. Neurol. 13, 593-614.

Austin, J.H., Balasubramanian, A.S., Pattabiraman, T.N., Saraswathi, S., Basu, D.K., Bachhawat, B.K. (1963), J. Neurochem. 10, 805-816.

Balasubramanian, A.S., Bachhawat, B.K. (1975), Biochim. Biophys. Acta 403, 113-121.

Barber, M., Brooksbank, B.W.L., Kuper, S.W.A. (1951), J. Pathol. Bacteriol. 63, 57-64.

Bartholomew, B., Dodgson, K.S., Matcham, G.W.J., Shaw, D., White, G.F. (1977), Biochem. J. 165, 575-580.

Batts, B.D. (1966), J. Chem. Soc. B. 551-555.

Baum, H., Dodgson, K.S. (1958), Biochem. J. 69, 573-582.

Baum, H., Dodgson, K.S., Spencer, B. (1958), Biochem. J. 69, 567-572.

Baum, H., Dodgson, K.S., Spencer, B. (1959), Clinica Chim. Acta 4, 453-455.

Beaven, G.H., Holiday, E.R. (1952), Adv. Prot. Chem. 7, 319-386.

Benkovic, S.J., Fedor, J.M. (1972), J. Amer. Chem. Soc. 94, 8928-8929.

Benson, J.R., Hare, P.A. (1975), Proc. Natl. Acad. Sci. 72, 619-622.

Bishayee, S., Bachhawat, B.K. (1974), Biochim. Biophys. Acta 334, 378-388.

Bleszynski, W.S. (1967), Enzymologia 32, 169-181.

Bleszynski, W., Lesnicki, A. (1967), Enzymologia 33, 373-389.

Bleszynski, W., Lesnicki, A., Lewosz, J. (1969), Enzymologia 37, 314-324.

Bleszynski, W.S., Roy, A.B. (1973), Biochim. Biophys. Acta 317, 164-171.

Blout, E.R., Fasman, G.D. (1958), Recent Advan. Gelatin Glue Res., Proc. Conf. Univ. Cambridge, p.122.

Burkhardt, G.N., Wood, H. (1929), J. Chem. Soc. 141-152.

Chen, R.F. (1967), Anal. Lett. 1, 35-42.

Chen, Y.H., Yang, J.T., Chau, K.H. (1974), Biochemistry, 13, 3350-3359.

Cloves, J.M., Dodgson, K.S., Games, D.E., Shaw, D.J., White, G.F. (1978), Biochem. J. 167, 843-846.

Coderch, R., Lluis, C., Bozal, J. (1979), Biochim. Biophys. Acta 566, 21-31.

Cohen, S.A., Pratt, R.F. (1980), Biochem. 19, 3996-4003.

Collins, J., Yamada, W., Worth, W., Austin, J. (1976), Adv. Exp. Med. Biol. 68, 225-232.

Colowick, S.P., Womack, F.C. (1969), J. Biol. Chem. 244, 774-777.

Cornish-Bowden, A. (1976), Principles of Enzyme Kinetics, Butterworth & Co. Ltd., London.

Cornish-Bowden, A., Eisenthal, R. (1978), Biochim. Biophys. Acta 523, 268-272.

Cuppett, C.C., Resnick, H., Canady, W.J. (1971), J. Biol. Chem. 246, 1135-1141.

Das, P.K., Bishayee, S. (1980), FEBS Lett. 111, 43-46.

Dean, M.F., Muir, H., Benson, V.F., Button, L.R., Batchelor, J.R., Bewick, M. (1975), Nature 257, 609-612.

Derrien, M. (1911), Bull. soc. chim. biol. 9, 110.

Dodgson, K.S. (1959), Enzymologia 20, 301-312.

Dodgson, K.S. (1961), Biochem. J. 78, 312-319.

Dodgson, K.S., Melville, T.H., Spencer, B., Williams, K. (1954), Biochem. J. 58, 182-187.

Dodgson, K.S., Rose, F., Spencer, B. (1957), Biochem. J. 66, 357-363.

Dodgson, K.S., Rose, F., Spencer, B., Thomas, J. (1957), Biochem. J. 66, 363-368.

Dodgson, K.S., Spencer, B. (1953a), Biochem. J. 53, 444-451.

Dodgson, K.S., Spencer, B. (1953b), Biochem. J. 55, 436-440.

Dodgson, K.S., Spencer, B. (1956a), Biochim. Biophys. Acta 21, 175.

Dodgson, K.S., Spencer, B. (1956b), Biochem. J. 62, 30P.

Dodgson, K.S., Spencer , B. (1957), Meth. Biochem. Anal. 4, 211-255.

Dodgson, K.S. Spencer, B., Thomas, J., (1953), Biochem. J. 53, 452-457.

Dolly, J.O., Dodgson, K.S., Rose, F.A. (1972), Biochem. J. 128, 337-345.

Draper, R.K., Fiskum, G.M., Edmond, J. (1976), Arch. Biochem. Biophys. 177, 525-538.

Dubois, G., Zalc, B., Le Saux, F., Baumann, N. (1980), Anal. Biochem. 102, 313-317.

Edelhoch, H., Perlman, R.L., Wilchek, M. (1968), Biochem. 7, 3893-3900.

Egami, F., Uchida, T. (1978), Seikagaku 50, 1297-1303.

Eto, Y., Rampini, S., Wiesmann, U., Herschkowitz, N.N. (1974), J. Neurochem. 23, 1161-1170.

Feitelson, J. (1969), Photochem. Photobiol. 9, 401-410.

Fisher, G., Jatzkewitz, H. (1977), Biochim. Biophys. Acta 481, 561-572.

Fisher, G., Jatzkewitz, H. (1978), Biochim. Biophys. Acta 528, 69-76.

Fluharty, A.L., Stevens, R.L., Millar, R.T., Kihara, H. (1974), Biochim. Biophys. Res. Comm. 61, 348-354.

Flynn, T.G., Dodgson, K.S., Powell, G.M., Rose, F.A. (1967), Biochem. J. 105, 1003-1012.

Foster, J.A., Bruenger, E., Rubin, L., Imberman, M., Kagan, H., Mecham, R., Franzdau, C. (1976), Biopolymers 15, 833-841.

Fowler, L.R., Rammler, D.H. (1964), Biochem. 3, 230-237.

French, A.P., Warren, J.C. (1967), Biochem. J. 105, 233-241.

Frieden, C. (1970), J. Biol. Chem. 245, 5788-5799.

Ginsberg, L.C., Di Ferrante, N. (1977), Biochem. Med. 17,80-86.

Gniot-Szulzycka, J. (1972), Acta Biochim. pol. 19, 181-190.

Gniot-Szulzycka, J. (1974), Acta Biochim. pol. 21, 247-254.

Goldstone, A., Koenig, H. (1973), Biochem.J. 132, 267-282.

Goldstone, A., Koenig, H. (1974a), Biochem. J. 141,527-535.

Goldstone, A., Koenig, H. (1974b), FEBS Lett. 39, 176-181.

Goldstone, A., Konecny, P., Koenig, H. (1971), FEBS Lett. 13, 68-72.

Graham, E.R.B., Roy, A.B. (1973), Biochim. Biophys. Acta 329, 88-92.

Guggenheim, E.A. (1926), Phil. Mag. Ser. VII 2, 538.

Guilbault, G.G. (1973), Practical Fluorescence, Theory Methods and Techniques, Marcel Dekker, Inc., N.Y.

Guilbault, G.G., Hieserman, J. (1969), Anal. Chem. 41, 2006-2009.

Gutfreund, H., Hammond, B.R. (1963), Nature 198, 667-670.

Harada, T., Spencer, B. (1964), Biochem. J. 93, 373-378.

Harada, T., Kono, K., Yagi, K. (1954), Mem. Inst.Sci. Ind. Res. Osaka Univ. Japan 11, 193-200.

Harzer, K., Benz, H.U. (1974), Hoppe-Seylers zeit.fur phys. chem. 355, 744-748.

Hayachi, K., Imoto, T., Funatsu, M. (1963), J. Biochem (T) 54, 381-387.

Herrmann, K., Diezel, W., Kopperschläger, G. Hofmann, E. (1973), FEBS Lett. 36, 190-192.

Hijazi, N.H., Laidler, K.J. (1973), Cdn. J. Biochem. 51, 806-814.

Hook, G.E.R., Dodgson, K.S., Rose, F.A., Worwood, M. (1973), Biochem. J. 134, 191-195.

Hovanec, J.W., Broomfield, C.A., Steinberg, G.M. (1977), Biochim. Biophys. Acta 483, 312-319.

Hussey, C., Orsi, B.A., Scott, J., Spencer, B. (1965), Nature 207, 632-634.

Jacobsen, C.F., Leonis, J., Linderstrom-Lang, K., Ottesen, M. (1958), Methods of Biochem. Anal. 4, 171-210.

Jaenicke, R. (1970), Pyridine Nucleotide Dependent Dehydrogenases, H. Sund, Ed., Springer-Verlag, Berlin, p.209.

James, G.T. (1979), Arch. Biochem. Biophys. 197, 57-62.

James, G.T., Austin, J.H. (1979), Clinica Chimica Acta 98, 103-111.

Jeffrey, H.J., Roy, A.B. (1977), Anal. Biochem. 77, 478-485.

Jerfy, A., Roy, A.B. (1969), Biochim. Biophys. Acta 175, 355-364.

Jerfy, A., Roy, A.B. (1972), Anal. Biochem. 49, 610-613.

Jerfy, A., Roy, A.B. (1973), Biochim. Biophys. Acta 293, 178-190.

Jerfy, A., Roy, A.B. (1974), Biochim. Biophys. Acta 371, 76-88.

Jerfy, A., Roy, A.B., Tomkins, H. (1976), Biochim. Biophys. Acta 422, 335-348.

Jevons, F.R., (1963), Biochem. J. 89, 621-624.

Karlsson, K.A., Samuelsson, B.E., Steen, G.O. (1968), Acta Chem. Scand. 22, 2723-2724.

Karlsson, K.A., Samuelsson, B.E., Steen, G.O. (1969), Biochim. Biophys. Acta 176, 429-431.

Kawiak, J., Sawicki, W., Miks, B. (1964), Acta Histochem. 19, 184-190.

Kean, E.L. (1968), J. Lipid. Res. 9, 319-327.

Kirschner, K., Eigen, M., Bittman, R., Voight, B. (1966), Proc. Natl. Acad. Sci. 56, 1661-1667.

Kushnir, T., Navon, G. (1975), Protein-Ligand Interactions, H. Sund, G. Glauer, Ed., Walter de Gruyter & Co., Berlin, p. 174.

Lakowicz, J.R., Weber, G. (1973), Biochem. 12, 4171-4179.

Lakshmi, S., Balasubramanian, A.S. (1980), Biochim. Biophys. Acta 614, 446-458.

Law, P.Y., Fisher, G., Loh, H.H., Herz, A. (1979), Biochem. Pharm. 28, 2557-2562.

Leder, I.G. (1980), Biochim. Biophys. Research Comm. 94, 1183-1189.

Lee, G.D., Van Etten, R.L. (1975a), Arch. Biochem. Biophys. 166, 280-294.

Lee, G.D., Van Etten, R. (1975b), Arch. Biochem. Biophys. 171, 424-434.

Logan, G.G., Warren, J.C. (1969), Biochem. J. 114, 707-710.

Loosemore, M.J., Cohen, S.A., Pratt, R.F. (1980), Biochem. 19, 3990-3995.

Lowenstein, J.M., Young, L. (1952), Biochem. J. 52, xxv.

Maengwyn-Davies, G.D., Friedenwald, J.S. (1954), Arch. Biochim. Biophys. 53, 29-43.

Mayers, D.F., Kaiser, E.T. (1968), J. Amer. Chem. Soc. 90, 6192-6193.

Mead, J.A.R., Smith, J.N., Williams, R.T. (1955), Biochem. J. 61, 569-574.

Mehl, E., Jatzkewitz, H. (1964), Hoppe-Seylers zeit. fur phys. chem. 339, 260-276.

Mehl, E., Jatzkewitz, H. (1968), Biochim. Biophys. Acta 151, 619-627.

Meyer, L.J., Becker, M.A. (1977), J. Biol. Chem. 252, 3919-3925.

Milsom, D.W., Rose, F.A., Dodgson, K.S. (1972), Biochem. J. 128, 331-336.

Milthorpe, B.K., Jeffrey, P.D., Nichol, L.W. (1975), Biophysical Chemistry 3, 169-176.

Moser, H.W., Sugira, M., Harbison, M.D., Williams, M. (1972), Advances in Experimental Medicine and Biology, Volk, B.W., Aronson, S.M., Eds., Plenum Press, New York, Vol. 19, p.429-450.

Mouttet, C., Fouchier, F., Nari, J., Richard, J. (1974), Eur. J. Biochem. 49, 11-20.

Munthe-Kaas, A.C., Berg, T., Seljelid, R. (1976), Exp. Cell. Res. 99, 146-154.

Nakae, Y., Ikeda, K., Azuma, T., Hamaguchi, K. (1972), J. Biochem. (T) 72, 1155-1162.

Neuberg, C., Kurono, K. (1923), Biochem. Z. 140, 295-298.

Nichol, L.W., Roy, A.B. (1964), J. Biochem. 55, 643-651.

Nichol, L.W., Roy, A.B. (1965), Biochem. 4, 386-396.

Nichol, L.W., Roy, A.B. (1966), Biochem. 5, 1379-1388.

Nicholls, R.G., Roy, A.B. (1971), Biochim. Biophys. Acta 242, 141-151.

O'Brien, J.F., Cantz, M., Spranger, J. (1974), Biochim. Biophys. Res. Comm. 60, 1170-1177.

Perlmann, G.E., Diringer, R. (1960), Ann. Rev. Biochem. 29, 151-182.

Perlmann, G.E., Longsworth, L.G. (1948), J. Amer. Chem. Soc. 70, 2719-2724.

Pertoft, H., Wärmegård, B., Hook, M. (1978), Biochem. J. 174, 309-317.

Pflumn, M.N., Beychok, S. (1969), J. Biol. Chem. 244, 3973-3989.

Porter, M.T., Fluharty, A.L., De La Flor, S.D., Kihara, H. (1972), Biochim. Biophys. Acta 258, 769-778.

Porter, M.T., Fluharty, A.L., Kihara, H. (1971), Science 172, 1263-1265.

Poulos, A., Beckman, K. (1980), Clin. Chim. Acta 101, 277-285.

Powell, G.M., Curtis, C.G., Dodgson, K.S. (1967), Biochem. Pharm. 16, 1997-2001.

Prosser, C.I., Roy, A.B. (1980), Biochim. Biophys. Acta 613, 130-139.

Quadri, S.F., Seib, P.A., Deyoe, C.W. (1973), Carbohydrate Res. 29, 259-264.

Rammler, D.H., Grado, C., Fowler, L.R. (1964), Biochem. 3, 224-230.

Rhodes, T.L., Stout, R.L., Edmond, J. (1976), Fed. Proc. Fed. Am. Soc. Exp. Biol. 35, 1632.

Roughton, F.J. (1954), Discuss. Faraday Soc. 17, 116-120.

Roy, A.B. (1953), Biochem. J. 55, 653-661.

Roy, A.B. (1956), Biochem. J. 62, 41-50.

Roy, A.B. (1958), Biochem. J. 68, 519-528.

Roy, A.B. (1960), Biochem. J. 77, 380-386.

Roy, A.B. (1971a), Biochim. Biophys. Acta 227, 129-138.

Roy, A.B. (1971b), The Enzymes, Vol.5, P.D. Boyer, Ed., Academic Press, London, p.1-19.

Roy, A.B. (1975), Biochim. Biophys. Acta 377, 356-363.

Roy, A.B. (1976), Aust. J. of Exper. Biol. and Med. Sci. 54, 111-135.

Roy, A.B. (1978), Biochim. Biophys. Acta 526, 489-506.

Roy, A.B. (1979), Biochim. Biophys. Acta 568, 103-110.

Roy, A.B. (1980), Ciba Symposium No.72, p.177-190,

Roy, A.B., Jerfy, A. (1970), Biochim. Biophys. Acta 207, 156-163.

Royer, G., Wildnauer, R., Cuppett, C.C., Canaday, W.J., (1971), J. Biol. Chem. 246, 1129-1134.

Rybarska-Stylinska, J., Van Etten, R.L., (1979), Biochim. Biophys. Acta 570, 107-117.

Sanghavi, P., Koenig, H. (1976), Biochem. J. 155, 725-728.

Schwartz, E.R., Ogle, R.C., Thompson, R.C. (1974), Arthr. Rheum. 17, 455-467.

Shapiro, L.J., Aleck, K.A., Kaback, M.M., Itabashi, H., Desnick, R.J., Brand, N., Stevens, R.L., Fluharty, A.L., Kihara, H. (1979), Ped. Res. 13, 1179-1181.

Shapiro, E., Nadler, H.L. (1975), J. Ped. 86, 881-884.

Shapiro, A.L., Vinuela, E., Maizel, J.V., Jr. (1967), Biochem. Biophys. Res. Comm. 28, 815-820.

Sherman, W.R., Stanfield, E.F. (1967), Biochem. J. 102, 905-909.

Shifrin, S., Kaplan, N.A. (1958), Proc. Natl. Acad. Sci. 44, 177-181.

Shifrin, S., Kaplan, N.A., Ciotti, M.M. (1959), J. Biol. Chem. 234, 1555-1562.

Shiga, T., Shiga, K. (1973), J. Biochem. (T) 74, 103-109.

Simpson, R.B., Kauzman, W. (1953), J. Amer. Chem. Soc. 75, 5139-5152.

Smith, J.N. (1951), J. Chem. Soc. 636, 2861-2863.

Solling, H., Esmann, V. (1977), Eur. J. Biochem. 81, 129-139.

Spencer, B. (1958), Biochem. J. 69, 155-159.

Spencer, B. (1959), Biochem. J. 73, 442-447.

Steiner, R.F., Greer, L., Bhat, R., Otan, J. (1980), Biochim. Biophys. Acta 611, 269-279.

Stevens, R.L., Fluharty, A.L., Skokut, M.H., Kihara, H. (1975), J. Biol. Chem. 250, 2495-2501.

Stevens, R.L., Fluharty, A.L., Killgrove, A.R., Kihara, H. (1976), Biochim. Biophys. Acta 445, 661-671.

Stevens, R.L., Fluharty, A.L., Killgrove, A.R., Kihara, H. (1977), Biochim. Biophys. Acta 481, 549-560.

Stevens, R.L., Hartman, M., Fluharty, A.L., Kihara, H. (1973), Biochim. Biophys. Acta 302, 338-344.

Stinshoff, K. (1972), Biochim. Biophys. Acta 276, 475-490.

Stoffel, W. (1971), Ann. Rev. Biochem. 40, 57-82.

Stumpf, D., Austin, J. (1971), Arch. Neurol. 24, 117-124.

Stumpf, D., Austin, J., Crocker, A., La France, M. (1973), Am. J. Dis. Child 126, 747-755.

Svedberg, T., Pedersen, K.O. (1940), The Ultracentrifuge, London, Oxford Univ. Press (Johnson Reprint Corp., New York, N.Y.).

Tallan, H.H., Bella, S.T., Stein, W.H., Moore, S. (1955), J. Biol. Chem. 217, 703-708.

ten Bosch, J.J., Rahn, R.O., Longsworth, J.W., Shulman, R.G. (1968), Proc. Natl. Acad. Sci. 59, 1003-1008.

Uchida, T., Egami, F., Roy, A.B. (1981), Biochim. Biophys. Acta 657 (in press).

Valenzuela, P., Bender, M.L. (1969), Proc. Natl. Acad. Sci. 63, 1214-1221.

Virden, R., Bristow, A.F., Pain, R.H. (1978), Biochem. Biophys. Res. Comm. 82, 951-956.

Vladimir, Yu.A., Zimina, G.M. (1965), Biokhimiya 30, 1105-1114.

Vlitos, A.J. (1953), Contrib. Boyce Thompson Inst. 17, 127-149.

Waheed, A., Van Etten, R. (1978), Anal. Biochem. 89,550-560.

Waheed, A., Van Etten, R.L. (1979), Arch. Biochem. Biophys. 194, 215-225.

Waheed, A., Van Etten, R.L. (1980a), Arch. Biochem. Biophys. 202, 366-373.

Waheed, A., Van Etten, R.L. (1980b), Arch. Biochem. Biophys. 203, 11-24.

Waley, S.G. (1980), Biochem. J. 185, 771-773.

Webb, E.C., Morrow, P.F.W. (1959), Biochem. J. 73, 7-15.

Weber, K., Osborn, M. (1969), J. Biol. Chem. 244, 4406-4412.

Weber, B., Rosenheck, K. (1964), Biopolymers Symp. 1, 333-341.

Wetlaufer, D.B., Edsall, J.T., Hollingworth, B.R. (1958), J. Biol. Chem. 233, 1421-1428.

White, A. (1959), Biochem. J. 71, 217-220.

Whitehead, J.E.B., Morrison, A.R., Young, L. (1952), Biochem. J. 51, 585-594.

Wilkinson, G.N. (1961), Biochem. J. 80, 324-332.

Wootton, J.F., Hess, G.P. (1962), J. Amer. Chem. Soc. 84, 440-448.

Worwood, M., Dodgson, K.S., Hook, G.E.R., Rose, F.A. (1973), Biochem. J. 134, 183-190.

Yamato, K., Handa, S., Yamakawa, T. (1974), J. Biochem. (T) 75, 1241-1247.

Yang, C-H., Srivastava, P.N. (1976), Biochem. J. 159, 133-142.

Yphantis, D.A. (1964), Biochem. 3, 297-317.

Yphantis, D.A, Waugh, D.F. (1956), J. Phys. Chem. 60, 623-629.

Zuckerman, N.G., Hagerman, D.D. (1969), Arch. Biochem. 135, 410-415.

LIST OF PUBLICATIONS

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1. "The Sulphatase of Ox Liver XXIII. The Nature of Substrate-Modified Sulphatase A", Connie I. Prosser, A.B. Roy, (1980), Biochim. Biophys. Act, 613, 130-139.

2. "Circular Dichroism Spectroscopic Studies of Native and Turnover-Modified Sulphatase A", Connie I. Prosser, David I. Marlborough, A.B. Roy (1980), Arch. Biochem. Biophys. 202, 661-663.

3. "Substrate-Modified Sulphatase A", C.I. Prosser, D.I. Marlborough, A.B. Roy, (1980), Proc. Aust. Biochem. Soc., 13, 31.

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THE SULPHATASE OF OX LIVER

XXIII. THE NATURE OF SUBSTRATE-MODIFIED SULPHATASE A

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Summary

An improved method is described for the preparation of milligram quantities of substrate-modified sulphatase A. The latter has the same molecular weight and the same ability to form a tetramer as has native sulphatase A. It has been shown that the modified enzyme prepared with nitrocatechol [^{35}S]sulphate as substrate contains 1 mol $^{35}SO_4^{2-}$ per mol enzyme and that any treatment which causes reversion of the modified enzyme to native enzyme is accompanied by the loss of the bound SO_4^{2-}. Dialysis of the ^{35}S-modified enzyme against a solution containing SO_4^{2-} causes a loss of $^{35}SO_4^{2-}$ with no change in the amount of modified enzyme in the preparation.

It has been shown that the activation of the substrate-modified enzyme by SO_4^{2-} does not lead to the formation of a third stable form of sulphatase A.

Introduction

The 'anomalous' kinetics of sulphatase A (aryl-sulphate sulphohydrolase, EC 3.1.6.1) were first described by Roy [1] for the sulphatase A of ox liver and were investigated in considerable detail by Baum et al [2] using the corresponding enzyme from human liver. The latter authors showed [3] that the 'anomalous' kinetics were the result of the enzyme being inactivated during its catalytic cycle and subsequently being reactivated by the reaction products, particularly by SO_4^{2-}. Further evidence for this explanation was provided by

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Abbreviation: PPO, 2,5-diphenyloxazole.

Nicholls and Roy [4]. Although it is now clear that the production of a substrate-modified enzyme is typical of the behaviour of sulphatase A functioning as an arylsulphatase, but not as a cerebroside sulphatase [5], there has been no indication of the nature of the change undergone by the enzyme in the presence of substrate. It appears to have been assumed to be conformational [6] because it is freely reversible at 37 °C in the absence of substrate [4,6]. Recent work in this laboratory [7] has, however, shown that the substrate-modified sulphatase of ox liver contains 1 mol SO_4^{2-} per mol enzyme. A similar finding has been reported by Waheed and Van Etten [8] with the sulphatase A of rabbit liver, although in this case there are 2 mol SO_4^{2-} bound per mol substrate-modified enzyme.

The present paper describes some of the physical and chemical properties of the substrate-modified sulphatase A of ox liver.

Experimental

Preparation of sulphatase A. Sulphatase A was prepared from ox liver by a slight modification of the method of Nichol and Roy [9]. Several different preparations were used; their specific activities ranged from 203 to 248 μmol · min^{-1} · mg^{-1}. These values are higher than those previously reported because they are based on computed values of the initial velocities determined in 0.1 M KCl (see below).

In the following, the molar concentration of sulphatase A is always expressed in terms of its monomer (M_r 107 000), whatever be the polymeric form of the enzyme dominant under particular experimental conditions.

Determination of sulphatase A activity. All determinations were made in a pH-stat (assembly PHM26-TT11-SBR2-ABU12, Radiometer Ltd., Copenhagen). Routine assays were carried out with 3 mM nitrocatechol sulphate as substrate in 0.1 M KCl, pH 5.6, at 37 °C. The volume of the reaction mixture was 10 ml.

Initial velocities were computed by fitting readings from the pH-stat recordings, at 8 s intervals over the first 3 min of the reaction, to the exponential function described by Roy [10].

The substrate-modified enzyme was activated by adding to the above reaction mixture 50 μl 0.6 M K_2SO_4. The velocity, 20—30 min after the addition of K_2SO_4, was obtained from the slope of the pH-stat recording, determined by linear regression of readings at 1-min intervals.

The fraction of substrate-modified sulphatase A in any preparation was calculated in either of two ways. In the first, the specific activity of the preparation, measured from v_0, expressed as a fraction of that of the corresponding native enzyme gave directly the amount of the latter remaining in the preparation. In the second, the ratio of v_0 to the velocity measured after the addition of SO_4^{2-} was used [4] to give the extent of the modification. Both methods assume [4] that the substrate-modified enzyme is devoid of activity in the absence of SO_4^{2-} and they give similar results. The first method was preferred because the enzyme concentrations were such that the reasonably high values of v_0 could be precisely determined. This was not so with the second method which was useful when only small amounts of enzyme were available.

Preparation of substrate-modified sulphatase A. Modified enzyme was

prepared by incubating sulphatase A (25 μg/ml) in 0.05 M nitrocatechol sulphate/0.1 M Tris-HCl, pH 7.5, at 37 °C for 30 min. In routine preparations, 50 ml reaction mixture containing 1.25 mg sulphatase A were used. After incubation the mixture was cooled to 4 °C and applied to a Sephadex G-25 column (25 × 3.5 cm) in 0.01 M Tris-HCl/0.15 M NaCl (pH 7.4) and eluted with the same buffer. In this and all subsequent steps the temperature was kept at 4 °C. The portion of the eluate containing the protein, determined by prior calibration of the column with bovine serum albumin, was then applied to a DEAE-Sephadex A-50 column (7 × 1 cm) equilibrated with the above buffer. After thorough washing with the same buffer, the enzyme was eluted with 0.05 M Tris-HCl/0.5 M NaCl, pH 7.4. 0.5-ml fractions were collected and the enzyme was located by the method of Baum et al. [11] which depends upon the activation of the substrate-modified enzyme by pyrophosphate. The fractions containing the modified sulphatase A were combined, the protein concentration determined spectrophotometrically ($A^{1\%}_{280nm} = 7.0$; see below), and then stored at −4 °C.

Fluorimetric determination of protein. When the concentrations of preparations of substrate-modified sulphatase A were too low to be determined spectrophotometrically, they were obtained by the method of Benson and Hare [12]. To 0.5 ml of a solution of sulphatase A (15—20 μg/ml) in 1 M NaCl were added 0.5 ml *o*-phthaladehyde reagent [12] and 1 min after mixing the resulting fluorescence was measured in an Aminco-Bowman spectrofluorimeter ($\lambda_{ex} = 340$ nm; $\lambda_{em} = 455$ nm). A standard emission value for native sulphatase A compared to bovine serum albumin was determined, and secondary standards of the latter were run simultaneously with each set of determinations. It was assumed that native and substrate-modified sulphatase A gave derivatives having the same fluorescence.

Synthesis of nitrocatechol [^{35}S]sulphate. Nitrocatechol [^{35}S]sulphate was prepared by the method of Flynn et al. [13]. To 1.5 g 4-nitrocatechol in 3.2 ml CS_2 and 3.7 ml *N*,*N*-dimethylaniline were added 5 mCi chloro[^{35}S]sulphonic acid (Radiochemical Centre, Amersham) mixed with 0.7 ml of redistilled chlorosulphonic acid. The reaction mixture was worked up in the usual way [13] to give approx. 0.7 g nitrocatechol [^{35}S]sulphate with a specific activity of about 0.1 Ci/mol. The purity of the product was verified spectrophotometrically.

Measurement of radioactivity. Radioactivity (^{35}S) was determined in a Packard Tri-Carb Liquid Scintillation Spectrometer, model 3255, using either Bray's scintillation fluid or a mixture of xylene and Triton X-114 (8.3 : 5, v/v) containing 0.5% 2,5-diphenyloxazole (PPO).

Equilibrium ultracentrifugation. This was carried out by the method of Yphantis [14], as previously described [15], in a Spinco Model E ultracentrifuge using an An-D rotor. The conditions were as follows. At pH 7.5 and 5 °C, 18 000 rev./min for 27 h; at pH 5.0 and 5 °C, 10 000 rev./min for 33 h; at pH 5.0 and 20 °C, 10 000 rev./min for 23 h. Molecular weights were computed by the standard method, assuming that the $\bar{v}$ of substrate-modified sulphatase A was the same as that determined for native sulphatase A, 0.715 [15].

Results

Isolation of substrate-modified sulphatase A

The method described above allowed the isolation of substrate-modified sulphatase A in mg quantities, the usual yield being about 80% in terms of protein and the final solution having a concentration of about 0.7 mg/ml. Typically, such preparations contained about 90% of substrate-modified sulphatase A, similar to those previously described [4].

When such a preparation was incubated for a second time with nitrocatechol sulphate, as described above, and the enzyme again isolated then the amount of substrate-modified sulphatase in the latter rose to about 97%. As little was to be gained by the use of such highly modified preparations, they were not routinely prepared and samples containing about 90% of substrate-modified sulphatase A were used in the experiments described below.

Stability of substrate-modified sulphatase A

The modified enzyme was apparently stable indefinitely when stored frozen or at temperatures close to 0°C. At 37°C and pH 7.4, it was much less stable and reverted to what was apparently native sulphatase A in a reaction having a $t_{½}$ of about 5 h. At 20°C and pH 7.4, the $t_{½}$ was about 60 h, and at pH 5.0 and 37°C it was about 30 h. From the rates of the reaction at 20 and 37°C, the activation energy for the reversion of substrate-modified sulphatase A to native sulphatase A at pH 7.4, was calculated to be about 6 kJ · mol^{-1}.

At pH 5.6, the optimum pH for the arylsulphatase reaction, the addition of 3 mM K_2SO_4 made little difference to the rate of reversion to the native enzyme but the addition of 3 mM 4-nitrocatechol did increase the rate, as shown in Table I. This increase did not occur when both K_2SO_4 and 4-nitrocatechol were present.

Activation of substrate-modified sulphatase A

Substrate-modified sulphatase A is activated by SO_4^{2-} in the presence of nitrocatechol sulphate [2—4,6] but nothing is known of the mechanism of this reaction, or of the nature of the activated enzyme. That the latter is not yet a third stable form of sulphatase A was shown as follows.

TABLE I

REVERSION OF SUBSTRATE-MODIFIED TO NATIVE SULPHATASE A

The substrate-modified enzyme (0.1 mg/ml) was incubated in 0.125 mM sodium acetate 0.125 mM NaCl, pH 5.6, at 37°C with the addition of K_2SO_4 or 4-nitrocatechol as required. At the appropriate time, the amount of native enzyme was determined by measuring v_0, as described in the text. The substrate-modified enzyme initially contained 13% of native enzyme.

Addition	Native enzyme (%)	
	3 h	6 h
None	13	15
K_2SO_4 (3 mM)	17	20
4-Nitrocatechol (3 mM)	24	36
K_2SO_4 + nitrocatechol	18	18

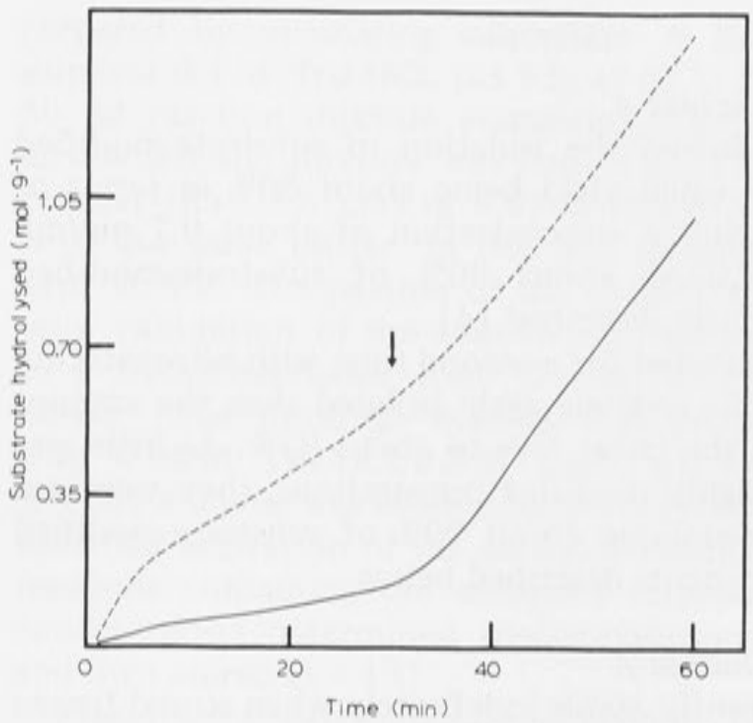

Fig. 1. Progress curves for the hydrolysis of 3 mM nitrocatechol sulphate by a standard preparation of substrate-modified sulphatase A (———) and by the same sample which had been activated by SO_4^{2-} and reisolated (- - - - -). In both cases 30 μmol SO_4^{2-} were added to the reaction mixtures at 30 min.

50 ml of a reaction mixture containing 25 μg of substrate-modified sulphatase A in 3 mM nitrocatechol sulphate and 0.1 M KCl were incubated in the pH-stat for 30 min at pH 5.6 and then made 3 mM in K_2SO_4 before incubating for a further 30 min. At the end of this time the enzyme was isolated in the usual way by chromatography on Sephadex G-25 and DEAE-Sephadex. The concentration of protein, determined fluorimetrically, showed a recovery of 18 μg (72%) sulphatase A. Progress curves for the hydrolysis of nitrocatechol sulphate by the recovered and original preparations of substrate-modified sulphatase A are shown in Fig. 1. It is clear that the progress curve with the recovered enzyme is similar to that with the original preparation of substrate-modified sulphatase A and that there is no indication that the activation by SO_4^{2-} has led to the formation of a stable form of sulphatase A different from the latter. If such a stable form had been produced the progress curve of the recovered enzyme would have been expected to be linear from the beginning of the reaction and the velocity to be equal to that attained after the addition of SO_4^{2-} prior to the reisolation of the enzyme. Also, no further activation would have been expected following the addition of SO_4^{2-} to the reaction mixture containing the recovered enzyme. It is, however, also clear from Fig. 1 that there has been, as expected [4], some regeneration of native sulphatase A during the reaction resulting in a increase in the initial velocity associated with the recovered enzyme.

Physical properties of substrate-modified sulphatase A

Ultraviolet absorption. The concentration of a sample of substrate-modified sulphatase A in 0.1 M Tris-HCl buffer, pH 7.5, was determined by differential refractometry, assuming a specific refractive increment of 0.184 ml · g^{-1}. Mea-

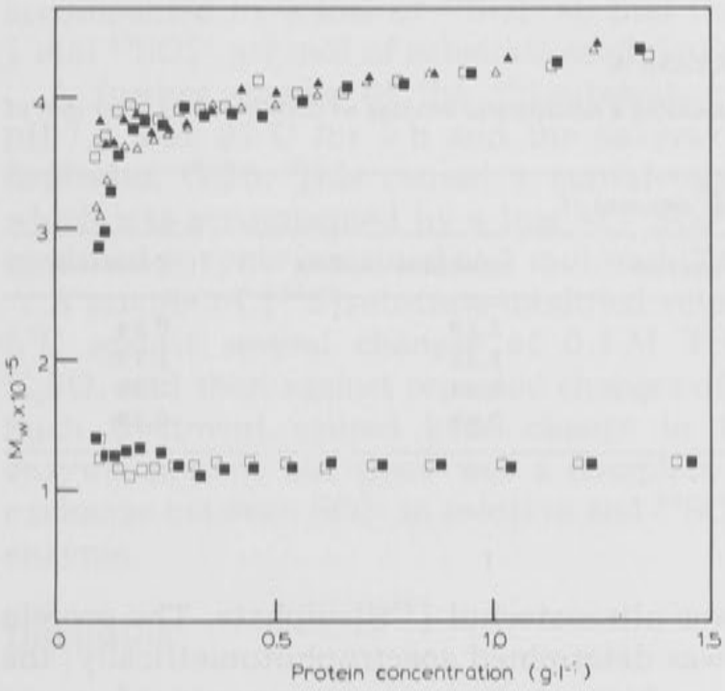

Fig. 2. Point-average values of the weight-average molecular weights of native and substrate-modified sulphatase A as a function of the total protein concentration during equilibrium ultracentrifugation at 5°C, $I = 0.1$. □,■; upper curve, substrate-modified sulphatase A after centrifugation for 31 and 33 h, respectively, at pH 5.0; lower curve, substrate-modified sulphatase A after 25 and 27 h centrifugation at pH 7.5. △,▲; native sulphatase A after centrifugation at pH 5.0 for 31 and 33 h.

surements in a Zeiss PMQ3 Spectrophotometer gave a value of 7.0 for $A^{1\%}_{280nm}$, the same as that for native sulphatase A [15].

There was no obvious difference between the ultraviolet absorption spectra of the native and substrate-modified forms of sulphatase A.

Molecular weight. In diethylbarbiturate buffer, pH 7.5, $\mu = 0.1$, at 5°C, substrate-modified sulphatase A had a molecular weight, determined by the Yphantis method [14], of 119 000 (range 116 000—121 000 in 4 measurements). This is within the range of values previously reported for native sulphatase A [15].

At high protein concentrations in acetate buffer, pH 5.0, $\mu = 0.1$, and at 5°C, substrate-modified sulphatase A existed predominantly as a tetramer but, as shown in Fig. 2, the tetramer dissociated at low protein concentrations. Native sulphatase A showed identical behaviour when examined under the same conditions (Fig. 2). In previous studies [15], no dissociation of the tetramer of native sulphatase A was noted during ultracentrifugation at pH 5.0 and 20°C. The latter experiments were therefore repeated, using the same sample of sulphatase A as was used to obtain the data in Fig. 2. No dissociation of the tetramer of sulphatase A occurred under these conditions, confirming its greater stability at higher temperatures. Its molecular weight was 448 000 which, as can be seen from Fig. 2, is the same as those of the tetrameric forms of both native and modified sulphatase A at pH 5.0 and 5°C.

^{35}S-Substrate-modified sulphatase A

Modification with nitrocatechol [^{35}S]sulphate. In these experiments, the substrate-modified enzyme was isolated by exactly the same procedure as

TABLE II

$^{35}SO_4^{2-}$ IN SUBSTRATE-MODIFIED SULPHATASE A

The value in the final column is obtained by assuming a nonspecific binding of 0.16 mol SO_4^{2-} per mol of total enzyme (see text).

Preparation	Fraction modified	$^{35}SO_4^{2-}$ per mol of		
		Total enzyme	Modified enzyme	Corrected
1	0.84	0.95	1.13	0.94
2	0.87	1.15	1.32	1.14
3	0.91	1.31	1.44	1.26
4	0.94	0.89	0.98	0.78

before, except that the substrate was nitrocatechol [^{35}S]sulphate. The protein concentration of the preparation was determined spectrophotometrically, the fraction of modified enzyme was obtained from measurements of v_0, as described above, and the amount of $^{35}SO_4^{2-}$ present was measured. The results, with four independent preparations of the substrate-modified enzyme, are shown in Table II. It is clear that approximately 1 mol of $^{35}SO_4^{2-}$ is associated with each mol of substrate-modified sulphatase A.

In control experiments, sulphatase A was incubated with $^{35}SO_4^{2-}$ under the conditions used for the preparation of the substrate-modified enzyme, except that no substrate was present. The concentration of SO_4^{2-} was 0.02 M, approximately that produced by the hydrolysis of nitrocatechol sulphate during the preparation of the substrate-modified enzyme. After incubation with $^{35}SO_4^{2-}$, the enzyme was recovered and examined as before. In two separate experiments the amounts of SO_4^{2-} bound to the native enzyme were 0.12 and 0.21 mol/mol, respectively.

Properties of ^{35}S-substrate-modified sulphatase A. A sample of ^{35}S-substrate-modified sulphatase A was activated by SO_4^{2-} during its reaction with nitrocatechol sulphate as has already been described for the unlabelled enzyme. As before, this treatment gave some reversion to the native enzyme and this was

TABLE III

$^{35}SO_4^{2-}$ IN SUBSTRATE-MODIFIED SULPHATASE A AFTER VARIOUS TREATMENTS

Details of the various treatments are given in the text. In each case, values are given for two independent experiments and are not corrected for any nonspecific binding of $^{35}SO_4^{2-}$.

Treatment	Fraction modified	$^{35}SO_4^{2-}$ per mol modified enzyme
Activated by SO_4^{2-}	0.61	1.10
	0.77	1.54
Incubated at 37°C	0.57	0.99
	0.52	1.07
Dialysed against SO_4^{2-}	0.83	0.0
	0.86	0.0

accompanied by a loss of $^{35}SO_4^{2-}$ so that the reisolated enzyme still contained 1 mol $^{35}SO_4^{2-}$ per mol of substrate-modified enzyme, as shown in Table III.

A further sample of the ^{35}S-substrate-modified enzyme was incubated at pH 7.5 and 37°C for 5 h and the enzyme recovered by chromatography on Sephadex G-25. This caused a considerable reversion to the native enzyme which was accompanied by a loss of $^{35}SO_4^{2-}$ so that the amount in the residual modified enzyme remained at 1 mol/mol (Table III).

A sample of [^{35}S]substrate-modified sulphatase A was dialysed for 3 days at 5°C against several changes of 0.1 M Tris-HCl, pH 7.4, containing 0.01 M K_2SO_4 and then against repeated changes of the buffer alone to remove K_2SO_4. Such treatment caused little change in the amount of substrate-modified enzyme present but there was a complete loss of $^{35}SO_4^{2-}$, indicating a facile exchange between SO_4^{2-} in solution and $^{35}SO_4^{2-}$ bound to the substrate-modified enzyme.

Discussion

The method for preparing substrate-modified sulphatase A requires little comment. It is similar to that previously described [4] except for the second chromatographic step, adsorption on DEAE-Sephadex, which allows an easy concentration of the eluate from the Sephadex G-25 column so that the present method is more easily adapted for the preparation of large amounts of the substrate-modified enzyme. The yield is about 80% and the preparations contain about 90% of substrate-modified enzyme. In accord with the demonstration [4] that there is an equilibrium between the native and substrate-modified forms of sulphatase A when these coexist with the substrate and the reaction products, it has been shown that the amount of residual native enzyme in the standard preparation of substrate-modified enzyme can be reduced to 2—3% by repeating the preparative procedure. Presumably even more highly-modified preparations could be obtained by further repetitions.

Attempts have been made to remove SO_4^{2-} from the reaction mixture, and so drive the equilibrium in favour of the modified enzyme [4], by having Ba^{2+} present. Such experiments have not been satisfactory because the very powerful adsorption of the enzyme to the precipitated $BaSO_4$ meant that the recovery of the modified enzyme was vanishingly small.

The reconversion of substrate-modified sulphatase A to native sulphatase A which occurs on incubation of the former at temperatures much above zero is not only of theoretical interest but also of practical importance. It means that investigations of the physical properties of the modified enzyme must be carried out at temperatures close to zero to prevent changes in the proportions of native and modified forms during the measurements. As has been shown in Table I, SO_4^{2-} has no effect on the reversion to native enzyme while 4-nitrocatechol stimulates it slightly.

The physical properties of the substrate-modified enzyme so far examined are not different from those of the native enzyme. Modification is not accompanied by any change in the molecular weight, the ability to form a tetramer at pH 5 or in the ultraviolet spectrum.

The dissociation of the tetrameric forms of both native and substrate-

modified sulphatase A which was obvious (Fig. 2) during equilibrium ultracentrifugation at pH 5 and 5 °C was unexpected and would not have been predicted from the apparent association constant for tetramerisation of the native enzyme, $3 \cdot 10^9\ l^3 \cdot g^{-3}$, found for this system by chromatographic methods [16]. The apparent association constant pertaining to the system in the ultracentrifuge was computed by the method of Milthorpe et al. [17] to be $0.5 \cdot 10^6\ l^3 \cdot g^{-3}$, much less than that previously reported [16]. This value gives a curve which fits the experimental points in Fig. 2 despite the fact that the method is not well suited to the investigation of the sulphatase A system where the degree of dissociation of the tetramer is slight and the extrapolation to $\Omega_0(r)$ is hazardous [17]. The reason for the discrepancy between the present results from equilibrium ultracentrifugation and the previous ones from chromatography [16] is not obvious but may lie in the very different concentration ranges required for the two techniques, 0.1—1 $g \cdot l^{-1}$ in the ultracentrifuge and 0.5—50 $mg \cdot l^{-1}$ in chromatography. The apparent association constant for the latter was computed on the assumption that the system was of a monomer and tetramer; this simplification [15,16] may become increasingly invalid at higher protein concentrations and unfortunately the method of Milthorpe et al. [17], which does not require such a simplification, cannot properly handle the present system. More detailed studies are required, but for the present purpose the conclusion that there is no obvious difference between the association behaviour of native and substrate-modified sulphatase A is justified.

The most important finding in the present work is that [^{35}S]substrate-modified sulphatase A contains 1 mol $^{35}SO_4^{2-}$ per mol enzyme. Strictly speaking, all that has been shown is that the enzyme contains one atom ^{35}S per mol enzyme, but it is most unlikely that the very stable sulphate group could have been altered under the experimental conditions used for the preparation of the modified enzyme. Although the enzyme-$^{35}SO_4^{2-}$ link is stable during the preparation, the bound $^{35}SO_4^{2-}$ is lost when the modified enzyme reverts to the native enzyme, and also by dialysis of the modified enzyme against SO_4^{2-}.

Nothing is known of the nature of the bound sulphate in the modified enzyme. Its most likely form would be a sulphate ester or related compound, for example, a sulphate ester ($R \cdot OSO_3^-$) of tyrosine, serine or threonine; a sulphamate ($R \cdot NH \cdot SO_3^-$) of an amino group or a ring nitrogen in histidine; or a thiosulphate ($R \cdot SSO_3^-$) of cysteine. Perhaps pertinent is the observation that the Arrhenius activation energy for the reversion of substrate-modified sulphatase A to native sulphatase A, that is, for the removal of the bound SO_4^{2-}, is approximately 6 $kJ \cdot mol^{-1}$. A similar value was given by Lee and Van Etten [6] for the reversion of the substrate-modified sulphatase A of rabbit liver, and it is therefore interesting that the activation energies for the acid-catalysed hydrolysis of sulphate esters range from 5.7—6.5 $kJ \cdot mol^{-1}$ [18]. On the other hand, attempts to prepare 'substrate-modified' sulphatase A by sulphation of the native enzyme, in aqueous solution, with pyridine-sulphur trioxide have not been successful; such treatment inactivates sulphatase A, but there is nothing to suggest that this is due to the formation of a 'substrate-modified' enzyme because it is not reactivated by SO_4^{2-}.

Further information on the nature of the bound sulphate will be difficult to obtain because the modified enzyme must be kept at temperatures close to

zero to prevent its reversion to native sulphatase A. This requirement effectively prevents the use of most of the chemical techniques available for the investigation of protein structure.

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References

1 Roy, A.B. (1953) Biochem. J. 55, 653—651
2 Baum, H., Dodgson, K.S. and Spencer, B. (1958) Biochem. J. 69, 567—572
3 Baum, H. and Dodgson, K.S. (1958) Biochem. J. 69, 573—582
4 Nicholls, R.G. and Roy, A.B. (1971) Biochim. Biophys. Acta 242, 141—151
5 Roy, A.B. (1979) Biochim. Biophys. Acta 568, 103—110
6 Lee, G.D. and Van Etten, R.L. (1975) Arch. Biochem. Biophys. 166, 280—294
7 Roy, A.B. (1980) Ciba Symposium No. 72, in the press
8 Waheed, A. and Van Etten, R.L. (1979) Arch. Biochem. Biophys. 195, 248—251
9 Nichol, L.W. and Roy, A.B. (1964) J. Biochem. 55, 643—651
10 Roy, A.B. (1978) Biochim. Biophys. Acta 526, 489—506
11 Baum, H., Dodgson, K.S. and Spencer, B. (1959) Clin. Chim. Acta 4, 453—455
12 Benson, J.R. and Hare, P.E. (1975) Proc. Natl. Acad. Sci. U.S.A. 72, 619—622
13 Flynn, T.G., Rose, F.A. and Tudball, N. (1967) Biochem. J. 105, 1010—1012
14 Yphantis, D.A. (1964) Biochemistry 3, 297—317
15 Nichol, L.W. and Roy, A.B. (1965) Biochemistry 4, 386—396
16 Jerfy, A., Roy, A.B. and Tomkins, H. (1976) Biochim. Biophys. Acta 422, 335—348
17 Milthorpe, B.K., Jeffrey, P.D. and Nichol, L.W. (1975) Biophys. Chem. 3, 169—176
18 Burkhardt, G.N., Ford, W.G.K. and Singleton, E. (1936) J. Chem. Soc. 17—25

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Circular Dichroism Spectroscopic Studies of Native and Turnover-Modified Sulfatase A

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In a recent communication, A. Waheed and R. L. Van Etten (1979, *Arch. Biochem. Biophys.* **195,** 248) showed that the sulfatase A of rabbit liver (arylsulfate sulfohydrolase, EC 3.1.6.1), which becomes inactivated as it catalyzes the hydrolysis of substrate, covalently incorporates ^{35}S from nitrocatechol [^{35}S]sulfate during this reaction and at the same time loses most of its secondary structure in solution. Circular dichroism spectra presented here for the native and turnover-modified forms of the sulfatase A of ox liver indicate no difference in the region of the spectrum below 240 nm associated with polypeptide backbone contributions or in the region from 350–250 nm associated with the side-chain chromophore transitions. In addition no differences were evident for the two forms of the ox liver enzyme from ultraviolet absorbance and fluorescence spectroscopy measurements. From these data we conclude that, in contrast to the situation with the rabbit enzyme, there is no loss of secondary structure associated with inactivation of ox liver sulfatase A in the course of enzymic catalysis.

In a recent communication, Waheed and Van Etten (1) showed that the loss of activity of the sulfatase A of rabbit liver in the course of catalysis was accompanied both by the covalent incorporation of ^{35}S from the substrate into the enzyme and by a loss of secondary structure which was detected by changes in the relevant CD spectra. The CD spectrum of the native enzyme was characteristic of a protein with some ordered secondary structure, whereas that of the turnover-modified enzyme was characteristic of a protein with little or no ordered secondary structure. This result is somewhat unexpected since the activity of the modified enzyme can be increased by the addition of sulfate in the presence of substrate, and also because the modified enzyme will revert quite rapidly to the native form at temperatures above 0°C. In the case of the rabbit enzyme the $t_{1/2}$ at 37°C is approximately 36 h (4). Although it is not impossible for the maximum conformational stability of the native enzyme to be at temperatures above normal ambient conditions, particularly if a covalently bound ligand is involved (5), such behavior is unusual.

We wish to report here a study of the CD spectra of native and turnover-modified sulfatase A from ox liver. The modified enzyme was isolated from a reaction mixture of 0.05 M nitrocatechol sulfate, 0.1 M Tris–HCl, pH 7.5, 25 μg/ml sulfatase A after a 30-min incubation at 37°C as previously described (2). The extent of modification was shown to be 90% by comparing the initial velocity, at pH 5.6, of the preparation with that of the native enzyme.

The circular dichroism spectra were measured on a Cary 60 spectropolarimeter equipped with cell-compartment thermostating. Specific ellipticities were expressed in deg cm^2 dmol^{-1} using a mean residue weight of 115 to calculate the mean residue molar concentrations. The two forms of the enzyme were studied at 10°C, to avoid any likelihood of conversion of turnover-modified enzyme to native enzyme, in solutions buffered at pH 5.0 with 0.07 M sodium acetate, $I = 0.1$, and pH 7.4 with 5 mM Tris–HCl, $I = 0.1$.

In the case of the ox liver enzyme both the native and turnover-modified forms had CD spectra characteristic of a partially ordered secondary structure (Fig. 1), as reported for the native enzyme of rabbit liver by Waheed and Van Etten (1) with, however, no discernible difference between the spectra. The $[\theta]_{220}$ values were in the range −10,000 to −11,000° (deg cm^2 dmol^{-1}) and the parameters obtained from analyzing the spectra by the method of Chen *et al.* (6) over the wavelength range 240 to 201 nm gave the values of 38% α-helix, 35% β-structure, and 27% random coil for the secondary structure of the enzyme.

In addition to the ellipticity arising from the backbone polypeptide chain, a shallow low-magnitude positive ellipticity occurs in the wavelength region 300 to 260 nm, presumably from an intrinsic side-chain or ligand chromophore. Again there is no discernible difference in this part of the CD spectrum between native and turnover-modified forms of the ox enzyme, suggesting little, if any, conformational

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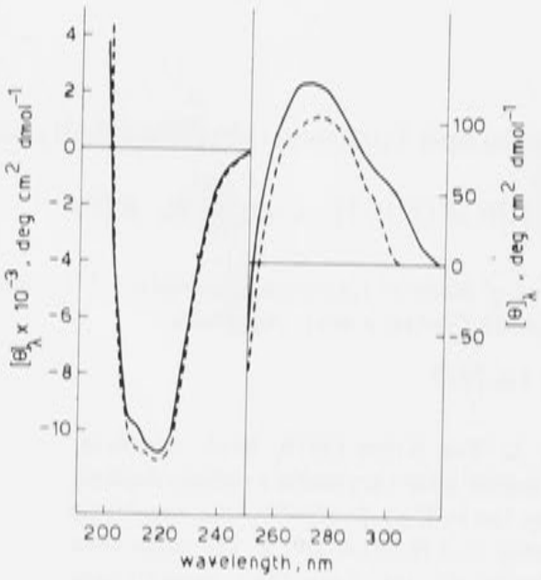

FIG. 1. Aqueous circular dichroism spectra of turnover-modified aryl sulfatase A (c = 0.06%) in 5 mM Tris–HCl, pH 7.4, I = 0.1 (——), and in 0.07 M sodium acetate, pH 5.0, I = 0.1 (– – –). The equivalent spectra for the native enzyme are indistinguishable from those in the figure. All the spectra were measured at 10°C.

disruption around chromophores such as aromatic side chains or disulfide bridges.

The turnover-modified enzyme preparation used assayed at 10% of the enzymic activity of native sulfatase A both before and after the spectra were taken. Similarly no change was found in the activity of the solutions of the native enzyme. On heating at 45°C for 30 min the activity of the modified enzyme preparation was restored to the extent of 37% that of native sulfatase A. However, as would be expected, no difference was apparent in the CD spectrum at that temperature over the 30-min time interval.

Ultraviolet absorption and intrinsic fluorescence spectra were also run using the same samples of ox liver sulfatase A. As with the CD spectra no difference was detected between the native and modified enzymes at either pH. The uv spectra between 200 and 350 nm showed the characteristic protein peak at 280 nm with some ripples occurring between 250 and 280 nm, indicating fine structure, and rose sharply below 250 nm (the samples were not kept in an oxygen-free environment). The fluorescence spectra, as expected, were characteristic of tryptophan having a single peak with its maximum at 325 nm.

Waheed and Van Etten (1) also reported that treatment of the rabbit liver enzyme with a large molar excess of pyridine-sulfur trioxide (reagent/protein = 4.4×10^5) brought about a loss of secondary structure and activity similar to that occurring during substrate modification. We were unable to prepare a solution of pyridine-sulfur trioxide in dimethylformamide of the concentration reported by Waheed and Van Etten (1) but treatment of the ox liver enzyme in aqueous solution with a suspension of the solid reagent (50 mg/ml, as described by Waheed and Van Etten) caused a loss of 50% of the enzyme activity. However, the enzyme obtained from this treatment could not be reactivated by SO_4^{2-} and is, therefore, not comparable to the turnover-modified enzyme. It is probable that the treatment of ox liver sulfatase A with pyridine-sulfur trioxide resulted in denaturation of the enzyme.

It is difficult to explain the differences between the present results and those of Waheed and Van Etten (1) because the sulfatases A of rabbit and ox livers are generally similar. The native enzymes have slightly different molecular weights but at pH 7.5, at which some of the CD measurements have been made, both exist as functionally active monomers with molecular weights of 107,000 and 147,000 for the ox (7) and rabbit (4) enzymes, respectively. At pH values of about 5, where CD measurements have also been made, the enzymes exist as tetramers and dimers respectively but as the change in pH makes little difference to the CD spectra the different degrees of polymerization can have no bearing on this problem.

The turnover-modified enzymes from the two species also have generally similar kinetic properties (2, 4) and, when isolated from reaction mixtures containing nitrocatechol [^{35}S]sulfate, contain one and two atoms of ^{35}S per molecule of ox (8) and rabbit (1) enzyme, respectively. They have been prepared under similar conditions, the main difference being that the pH was 6.0 in the case of the rabbit enzyme (1) and 7.5 in that of the ox enzyme. This cannot account for the different results. There is nothing to suggest that different forms of turnover-modified ox sulfatase A are obtained at different pH values (9) and previous work (Roy, unpublished observations) has shown that the modified ox enzyme produced at pH 6.0 also had a CD spectrum indistinguishable from that of the native enzyme. A pH of 7.5 was used in the present work simply because of the higher yields of turnover-modified enzyme. The conditions under which the CD spectra were measured were also comparable except that the temperature was 10°C in the case of the ox enzyme and 37°C in the case of the rabbit enzyme. Although the latter conditions would favor the reversion of the turnover-modified to the native enzyme (2, 4) this cannot account for the different results.

Perhaps the answer lies in the recent studies of Waheed and Van Etten (10) which have shown that the turnover-modified form of the sulfatase A of rabbit liver is a very fragile protein whereas the corresponding enzyme from ox liver is quite stable. It may be, therefore, that there are unsuspected differences between the sulfatases A of ox and rabbit livers, particularly between their turnover-modified forms, and that the spectra measured by Waheed and Van Etten refer not to the turnover-modified form of rabbit liver sulfatase A but to some derivative thereof.

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REFERENCES

1. WAHEED, A., AND VAN ETTEN, R. L. (1979) *Arch. Biochem. Biophys.* **195**, 248–251.
2. NICHOLLS, R. G., AND ROY, A. B. (1971) *Biochim. Biophys. Acta* **242**, 141–151.
3. STINSHOFF, K. (1972) *Biochim. Biophys. Acta* **276**, 475–490.
4. LEE, G. D., AND VAN ETTEN, R. L. (1975) *Arch. Biochem. Biophys.* **166**, 280–294.
5. TANFORD, C. (1968) *Advan. Protein Chem.* **23**, 121–282.
6. CHEN, Y.-H., YANG, J. T., AND CHAU, K. H. (1974) *Biochemistry* **13**, 3350–3359.
7. NICHOL, L. W., AND ROY, A. B. (1965) *Biochemistry* **4**, 386–396.
8. PROSSER, C. I., AND ROY, A. B. (1980) *Biochim. Biophys. Acta*, in press.
9. ROY, A. B. (1979) *Biochim. Biophys. Acta* **568**, 103–110.
10. WAHEED, A., AND VAN ETTEN, R. L. (1980) *Arch. Biochem. Biophys.* **202**, 366–373.